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The development of a quantitative hydrogenation method; the use of this and other methods in following the progress of oxidation in milk fat

Robert H. Thomas
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THE DEVELOPMENT OF
A QUANTITATIVE HYDROGENATION METHOD;
THE USE OF THIS AND OTHER METHODS IN
FOLLOWING THE PROGRESS OF OXIDATION IN MILK FAT

by

Robert H. Thomas

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Food Technology (Dairy Chemistry)

Approved:

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In Charge of Major Work

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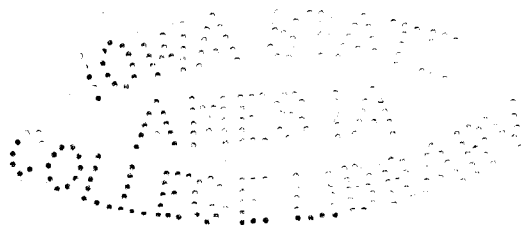
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Iowa State College
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I. INTRODUCTION

While milk is not a perfect food, as some people would have us believe, it does more nearly approach perfection than do most other foods. Milk falls short of perfection not only in its deficiency of several trace nutrients (for example, iron, copper and certain vitamins) but also in its perishability. Man, in his ceaseless quest for a better food supply, has sought a means to preserve milk, that it might be available when and where it is desired. Certain methods have been devised and used to varying extents for this preservation.

Without going into a philosophical discussion of the merits of such an attitude, it can be stated that man will reject, at least to a large degree if not entirely, food which has a disagreeable flavor. Admittedly, however, man can become accustomed to, or can tolerate, certain flavors which he once rejected. Nevertheless, if a preserved milk carries with it a foreign flavor it will not be consumed in great quantity by the majority of the people.

To date, the three general methods for the preservation of milk for extended periods are (a) heat treatment in combination with the use of sealed containers, (b) freezing, including holding in the frozen state and (c) dehydration. The heat

treatment necessary to preserve milk for more than just a few days imparts a characteristic flavor which a large number of people find undesirable. It is outside the scope of this thesis to discuss the mechanism of the formation of this flavor other than to say that it is not entirely understood but is believed to be associated in some way with one or more of the milk proteins.

Satisfactory preservation of milk by dehydration would mean an accompanying weight and volume reduction, a factor which becomes important when transporting and storing milk. When non-fat milk is dehydrated, it is possible to obtain a product which is acceptable to a large number of people and which retains this acceptability for a considerable time. To date, this generally has not been possible with whole milk, primarily because of the rather rapid development of an oxidized flavor in the lipid fraction of the product. Actually, this flavor is not limited to dehydrated dairy products. In some products, as for example, fluid milk, its appearance seems more prevalent as the bacteriological quality of the milk is improved. It has not yet been proven whether this is a result of a change in susceptibility or because other defects previously present had masked the flavor.

The time required for the development of oxidized flavor is quite variable. No method exists at this time for the prediction of the time which will be required for the flavor

to develop, although many people have attempted to devise a satisfactory method. Some of the most commonly used methods involve accelerated deterioration through high temperature storage. Even the mechanism by which oxidized flavor is produced is not clearly understood. The problem of fat oxidation has been studied by many researchers working with both edible and non-edible fats and oils. Other research workers have studied other products which undergo similar oxidative deterioration. An appreciable array of methods has been devised purporting to measure the degree of oxidation. None of these shows the desired degree of correlation with the one definite indication of deterioration, the presence of undesirable flavors and odors. Furthermore, none shows definite indication of developing deterioration until after the defect becomes obvious by organoleptic indications.

Studies on related materials have indicated that (other factors being equal) the greater the degree of unsaturation of a fat the greater should be its rate of deterioration. In certain dairy products the other factors are not equal. This is indicated by the more frequent occurrence of oxidized flavor in market milk during the winter than in the summer when there is a greater degree of unsaturation.

A decrease in the unsaturation of fats as measured by various halogen addition procedures indicates that the ethenoid linkages of the fat are involved in the oxidation. The

results of the various halogen addition procedures have been criticized since all of them do not give the same results. Even the results by a given reagent vary so much with reaction conditions that it is doubtful if any give a true measure of unsaturation. It was believed that hydrogen should (under the influence of a catalyst) add quantitatively to the double bonds. It should also react with any peroxides present. Therefore, the amount of hydrogen which will react with a given amount of fat should increase as the fat deteriorates. A study of the literature revealed no hydrogenation method considered satisfactory for the study of oxidation.

This study was undertaken with the above considerations in mind. The objectives of the study were (a) to develop a quantitative hydrogenation procedure for fats, (b) to determine whether or not the oxygen uptake during the useful life of a fat could be measured from the iodine and hydrogenation iodine values, (c) to compare changes in hydrogenation iodine value with changes in peroxide development, tocopherol degradation, carbonyl formation and organoleptic evaluation of milk fat and (d) to study the effect of the season of production and storage temperatures on the results obtained by the methods listed in (c). This third objective was an attempt to find both a method of accurately determining the degree of oxidation which had previously occurred and a method of predicting the rate at which oxidation would

subsequently occur. It was also hoped that the results of this study would give a greater insight into the mechanism of fat oxidation.

The term milk fat is used throughout the experimental portion of this thesis to refer to the fat obtained by melting, centrifuging and filtering the butter obtained by churning cream. This fat is considered to contain the triglyceride and non-saponifiable portions of the lipid fraction but not to include the phospholipids.

II. REVIEW OF LITERATURE

A. Autoxidation

Because unsaturated fats and fatty acids react spontaneously with oxygen, the term autoxidation has often been applied. Regardless of whether the term is completely justifiable on technical grounds, it will be used here to mean "oxidation by atmospheric oxygen" including that dissolved in the oil, fat or fatty acid in question. The scope of the reactions included within this term are considered to mean not only the original union with oxygen, but includes also such other changes as may be a result of this original reaction. The term thus encompasses certain intramolecular rearrangements, intermolecular reactions, chain scission and in some cases polymerizations.

1. Relationship to flavor

"Oxidized flavor" is a term that has been used very loosely in connection with milk and other dairy products. It has been known by a number of more descriptive terms such as "cappy," "cardboard," "oily," "metallic," "tallowy" and "fishy." There is some disagreement as to whether or not these are all merely different stages in the same type of

deterioration. Greenbank (79, p. 913) states that

The so-called tallowy flavor which develops in fluid milk is not the same as the tallowy flavor which develops in dried milk. The former generally is believed to be caused by an oxidation of the phosphatides, while the latter is caused by an oxidation of the glycerides.

This same author had earlier (78) concluded that the oxidized flavor of milk is caused by a mild oxidation of some minor constituent or constituents. He represented the oxidation schematically as follows:



He believed that all the major constituents of milk are relatively stable to mild oxidizing conditions, especially those which are known to affect flavor formation, and therefore considered casein, albumin, lactose and the glycerides of fat eliminated as precursors of the oxidized flavor.

Henderson and Roadhouse (92) measured the influence of various factors on the susceptibility of milk fat to oxidation. The fat used in their experiments was prepared by churning the cream and melting and filtering the fat. Sommer (195) believes that butter oil, produced by separation rather than by the evaporation of moisture from butter, is completely devoid of phospholipids. (It might be noted here that the materials examined by these authors were prepared in a manner similar to that used in the present study.)

There is a considerable amount of laxity in the use of terms in describing oxidized and related flavors, with the result that it is occasionally difficult to know whether a flavor described by an author is being attributed to autoxidation, and if so what compound or material is considered oxidized. For example, Musset et al. (158) speak of whole milk powder undergoing a flavor deterioration either during manufacture or shortly thereafter and state that the definition of the defect includes such terms as "typical of whole milk powder," "heated," "like coconut," "stale" and various others. They remind the reader that this defect should not be "confused with oxidized flavor" in dairy products since it is detectable in best quality whole milk powders long before the true oxidized flavor is normally evident. However, they continue (p. 299) by quoting Bailey (8) as follows:

Flavor reversion in fats is probably defined most satisfactorily as the appearance of objectionable flavor from less oxidation than is necessary to produce true oxidative rancidity.

Thus it seems that while some of these flavors may be caused by less drastic oxidation than others, there is, nevertheless, a problem of oxidation involved. The work of Musset et al. (157, 158) does not rule out the possibility of phospholipids being the precursors of the undesirable flavor in dry milk but they seem to imply that it might be the glyceride fraction,

for they state that the solid fractions (obtained by hydraulic pressing) having a much lower iodine number were all relatively stable with regard to flavor deterioration while the liquid fractions deteriorated rapidly.

Reinart (176) in discussing oxidized flavor finds oxidation to be greatest in samples having the highest unsaturation and concludes that oxidation is related to the concentration of highly unsaturated acids in the C_{20} - C_{22} series, thus being a problem of glyceride oxidation. Holm (101) states that fats of different degrees of unsaturation oxidize at different rates and have different oxygen absorption requirements to render them inedible, again pointing to the glyceride fraction, and in particular, the unsaturated glyceride fraction, as the precursor of oxidized flavor.

Greenbank (80) discusses oxidized flavor as being the result of an intermediate oxidation product, but in this case he does not (as he did in other references cited above) state what material he considers to be the precursors.

Pont (170), in a study of milk exposed to sunlight, found peroxide values of 0.30-2.00 meq. per kg. of fat in milks exposed long enough to cause a flavor change. In these cases the fat was "oily-tallowy" in flavor.

Some of the oxidized flavors in fresh milk, such as "chalky" and "chalky-to-soapy-tallowy" were considered by Krukovsky (117) to be associated with the milk plasma (skim

milk) while metallic and metallic-to-fishy flavors were associated with deterioration of the fat globule membrane and oxidation-sensitive fat respectively.

Mulder et al. (156) consider the oxidation to take place in the boundary of the fat phase or in the water phase of cream.

Further evidence that the oxidized flavor is a result of changes in the fat comes from Keeney and Doan (110, 111, 112) who distilled oxidized milk fat and consider that the pre-dominant odor compounds in oxidized milk fat were ketones and who further consider that they were able, by mixing the ketone and noncarbonyl neutral fraction in proper proportions, to simulate the flavor of oxidized milk.

Several investigators, including Roland et al. (182) and Roland and Trebler (183) have found that the tendency toward occurrence of oxidized flavor is a function of the fat content. However, Roland and Trebler found that recombination of skim milk and cream resulted in a reduced susceptibility to oxidized flavor and considered that this might be due to removal of lecithin or related substances or to changes in their distribution between the fat and aqueous phases.

In a study of oxidized flavor in strawberry ice cream, by Bird, Ross, and Iverson (14) the iodine numbers of the fats showed a tendency toward the greatest drop in those samples developing the flavor defect most rapidly.

Very extensive reviews on oxidized flavor in dairy products have been published by Brown and Thurston (27) and Greenbank (79).

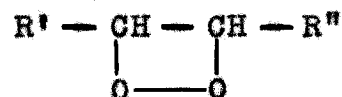
A study of the references cited would seem to leave little doubt that the precursor of the oxidized flavor is the lipid fraction of the dairy product, although there is not such general agreement as to just what constituent in this phase is responsible. While some workers favor the view that one or more of the phospholipids is responsible, others look to the glycerides, particularly those having unsaturated acid moiety, as being the oxidizable substrate. It must be pointed out that (a) the material examined in this study was obtained by churning and therefore would not be considered to contain phospholipids and (b) the material published with regard to the mechanism of the oxidation reaction seems in the main to deal with the reactions of unsaturated fatty acids.

2. Theories regarding the method of original attack by oxygen

Before one can understand the exact mechanism of the process of autoxidation, it is necessary to know where the reaction takes place and what is the nature of the first product of the process. Markley (133) attributes the first observation of autoxidation of a carbon-to-carbon double bond to Schönbein (187) in 1858. Since that time a number of hypotheses have been proposed, the evidence for some being rather indirect. Some of the hypotheses differ by reason of the fact that a compound considered a primary product by one person is considered by another to be a secondary material

formed from the primary product.

a. Cyclic peroxide formation. Various workers (57, 156, 198 and others) cite works of Bach (7) and of Engler and Weissberg (51, 52) as beginning modern theories of autoxidation. They proposed that a molecule of oxygen was added to the double bond with formation of a cyclic peroxide of the formula



However, most of the evidence purported to substantiate this theory has been indirect. Goldschmidt and Freudenberg (75) studied the autoxidation of pure linolenic acid and methyl linolenate by a determination of the double bond content (Wijs iodine method) of the original and autoxidized products. Comparison of the two values showed that one double bond disappeared for each molecule of oxygen consumed. It must, however, be remembered that possibly some of this oxygen might have gone into secondary oxidation products. In addition, they considered that the residual iodine values plus the hydriodic acid peroxide numbers were essentially equal to the iodine value of the original material. Their results indicate an iodine value of 274 for linolenic acid before oxidation and a value of 285 calculated from iodine value and peroxide value determined after oxidation.

Franke and Jerchel (69), in a study of autoxidation of oleic, ricinoleic, linoleic and linolenic acids, measured oxygen uptake, peroxide oxygen and iodine number. Values were plotted as per cent absorbed oxygen showing as peroxide oxygen, absorbed oxygen as per cent of theoretical absorption (on basis of one molecule oxygen per double bond) and iodine number as per cent of the experimentally determined value of the original material. On the assumption that one mole of oxygen is equivalent to the loss of one double bond, the theoretical curve connecting 100 per cent iodine number with 100 per cent oxygen absorption should be a straight line. These authors found their data to fall upon this theoretical line to about 25 per cent oxygen absorption with oleic and ricinoleic acids and to about 60 per cent with linoleic and linolenic acids. Beyond these percentages, the iodine number decreased less rapidly than indicated by the theory. However, the relationship between the per cent oxygen as peroxide and the oxygen absorption showed that as greater amounts of oxygen were absorbed, progressively less of it appeared as peroxide; there must have been considerable side or secondary reactions even before the iodine number-oxygen absorption curve deviated from theoretical. These studies were made using $\text{Co}(\text{NO}_3)_3$ as a catalyst and may not, therefore, be exactly comparable to results without this catalyst.

In a somewhat similar work on the rate of reaction of oleic acid with oxygen, Henderson and Young (93) plotted the per cent oxygen remaining in the form of titratable peroxides against moles of oxygen absorbed per mole of acid. Extrapolation of the curve to zero oxygen absorption indicated to them that peroxide formation was the initial reaction. According to Markley (133) the same results should be expected with a hydroperoxide. Although Henderson and Young did not so state, it is presumed that they did not mean hydroperoxide, but rather cyclic peroxide as evidenced by their discussion of loss of unsaturation. The fraction of oxygen absorbed and remaining as peroxide is a function of the moles of oxygen absorbed per mole of oleic acid. They consider that further evidence of peroxide formation as the initial reaction was found by plotting the double bond destruction against moles of oxygen absorbed per mole of acid. A figure is given purporting to show that as oxygen absorption approaches zero the double bond destruction is proportional to oxygen absorbed. However, this curve does not show at any place one double bond destroyed per molecule of oxygen.

Franke and Mönch (70) studied hydrogenation values of linoleic and linolenic acids which had been autoxidized to varying degrees. In no case did the amount of hydrogen absorbed exceed 100 per cent (based on theoretical value for pure fatty acids); hydrogenation was considered complete when

the iodine values of the hydrogenated products were zero. These authors felt that according to their hypothesis of cyclic peroxide formation the hydrogenation value of the oxidized material should not differ materially from that of the pure acids. They consider their results as substantiating this. However, it seems possible that if half or slightly more of the peroxides had reacted with double bonds in the manner suggested by Farmer *et al.* (57) this could account for the results obtained.

That the mode of attack of oxygen may depend upon temperature was pointed out by Atherton and Hilditch (6) in 1944. While they consider that autoxidation at ordinary temperature involves conversion of an alpha-methylene group into a hydroperoxide, they find that at 120°^a the results point to an almost complete lack of hydroperoxide, the action apparently taking place at an ethenoid group. They also found that the drop in iodine value of methyl oleate autoxidized at 20° was greater than that required for exclusive formation of hydroperoxide groups, but only about half that which would result from union of oxygen at the double bond. The following year Gunstone and Hilditch (84) confirmed the results at 20° and found the hydroperoxide formation process to be active at 100°

^aUnless otherwise specified all temperatures listed throughout this thesis are Centigrade temperatures.

to 120°; in addition, direct attack on the ethenoid bond seemed to occur.

After studying the products resulting from methyl linoleate autoxidation (1 mole oxygen per mole ester), bromination, permanganate oxidation and debromination, Toyama and Matsumoto (208) concluded that the majority of the autoxidation products are compounds in which the double bond at the C₉ atom is attacked by oxygen.

That the matter is not a simple one to which a single all-inclusive formula can be applied has been confirmed by many workers.

Mukherjee (146) believes that the initial step in autoxidation is the formation of a cyclic peroxide but that subsequent to the formation of a small quantity of this peroxide, the attack is by hydroperoxide formation, the energy liberated in cyclic peroxide formation being able to remove hydrogen from the alpha-methylene groups.

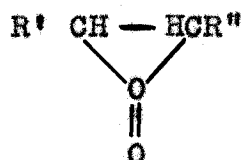
On autoxidation of 1,3- and 1,4-multiple unsaturated oxygen active long chain fatty acids, Treibs and Rothe (209, 210) believe that the double bonds are saturated with oxygen with formation of peroxides and not hydroperoxides as primary products. However, their studies of the oxidation products of oleic acid convince them that with this acid hydroperoxide is formed with the double bond migrating through the molecule.

Farmer and Sundralingam (59) consider that there is little direct evidence supporting cyclic peroxide formation beyond (a) the unsaturation of the autoxidized substance generally decreases progressively (though not in quantitative correspondence) as oxygen uptake increases and (b) that frequently the reaction ends in hydroxylation of the double bond to yield a 1,2-glycol, or in complete scission at the double bond. They seem to feel that this hypothesis does not satisfactorily account for the differences in products found, the differences in reaction rates and other differences that result from changing reaction conditions. These men and their co-workers (57) feel that disappearance of olefinic unsaturation rarely keeps pace with oxygen incorporation.

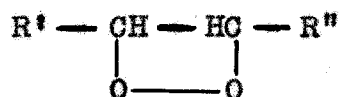
On the assumption that one molecule of oxygen saturates one double bond to form a cyclic peroxide, Paschke and Wheeler (167) have calculated from the decrease in iodine number during peroxidation, a "theoretical peroxide" value. Using distilled esters of soybean oils, they calculated that if all double bonds were converted to peroxide the theoretical peroxide value would be 9000 meq. per kg. Curves were presented showing that at 100° the determined and theoretical values coincide only to values of about 500 to 600 while the maximum peroxide value was about 750. At 75° these values were 1000 and 1700 respectively and 1700 and 2400 at 55°. At 35° and 15° they are about 2600 and 3100. They found little change in

unsaturation, hydroxyl number and acid number with peroxide decomposition.

b. Moloxide. In 1925 Staudinger (197) proposed that the cyclic peroxide was not the first but the second step in the autoxidation of ethylenic compounds. He assumed that the original addition would give a compound with the formula



which would then undergo change to a cyclic peroxide of structure

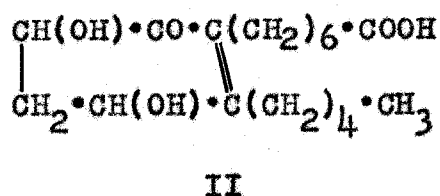
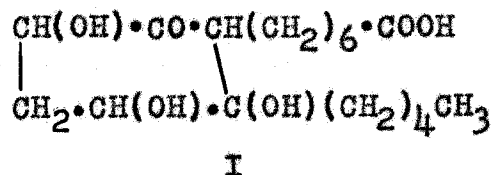


He suggested the name moloxide for the former and considered that the latter should be referred to as a peroxide.

It is well to note that Morrell and Davis (138) speak of a moloxide which they say may be of peroxidic or non-peroxidic nature. The former is a cyclic peroxide while the latter is a ketohydroxy form or its dihydroxy tautomeride.

c. Ketohydroxy-dihydroxy compounds. Ellis (46, 47) developed a hypothesis that linolenic acid upon autoxidation yields a compound with either three ketol groups, or, more likely, two ketol groups and one enediol, the latter at C₁₂-C₁₃. Linoleic acid is said to oxidize to a compound with two enediol groups which may undergo ring formation to give either

cyclohexanetriolone (I) or cyclohexanediolone (II).



In support of this a compound with the equivalent weight and hydroxyl content of II was said to have been isolated while it was considered that I might likewise exist. Goldschmidt and Freudenberg (75) had earlier found no evidence of hydroxyl groups after an uptake of two atoms of hydrogen per peroxide group formed.

Morrell and co-workers (137-144) used alpha- and beta-eleostearic acids; frequently a maleic anhydride adduct of these acids was employed. The maleic anhydride adduct of the beta form (137) showed a peroxide group in the aliphatic chain at C₁₃-C₁₄; a tautomeric equilibrium mixture of keto-hydroxy and di-hydroxy forms at the hydrobenzene ring double bond. The alpha adduct had a keto-hydroxy structure in the aliphatic chain at C₉-C₁₀ while the olefinic linkage in the ring did not oxidize.

Further study of beta-eleostearin showed (141) that the first product formed is a diperoxide (the double bond at

C₁₁-C₁₂ was not oxidized) which changed to a monoperoxyketo-hydroxy glyceride, the group nearer the ester linkage changing. Treatment of the methylated acid by hydrogen with palladium causes reduction of the peroxy group to an epoxide and saturation of the double bond nearest the ester group, leaving the middle double bond unaltered.

It would seem that the ketohydroxy form should be able to change rather freely into the dihydroxy (actually an enediol) form and apparently does so in the beta adduct. However, the alpha adduct upon oxidation gave a product which had at the "near" linkage a ketohydroxy but no dihydroxy structure (139). Hydrogenation of adducts of both alpha and beta acids resulted in the reduction of both ring and chain double bonds and changed peroxide to monoxide.

Upon heating the polymeride of the maleic anhydride adduct of the beta glyceride to 100-110° C., a decrease in peroxide content and an increase in the hydrogen percentage were noted (140). These changes were attributed to a degradation of peroxide to monoxide. In studying the properties of the ketol group, a mixture of 9-hydroxy-10-ketostearic and 10-hydroxy-9-ketostearic acids was found (142) to absorb oxygen readily. Enolization of ketol groups is said (144) to be responsible for the discrepancy between Wijs and Hübl iodine values.

Fahrion (53) considered that a cyclic peroxide was formed as the primary step preceding formation of ketchydroxy or dihydroxyethylenic configurations.

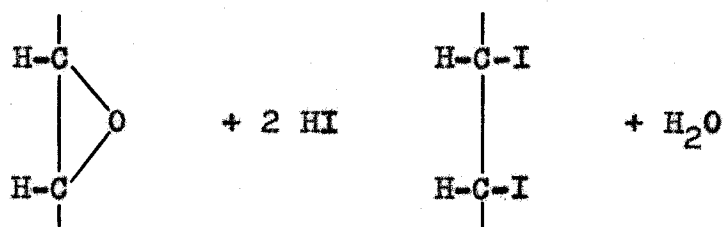
Farmer and Sundralingam in 1942 (59) have criticized the suggestion that a cyclic peroxide is first formed and then changed to ketol or enediol since it is not able to account for the formation of saturated 1,2-glycols. They seem to have overlooked the fact that Browne had about 17 years earlier (29) proposed a theory whereby a cyclic peroxide might be converted to a saturated glycol. He suggested that a cyclic peroxide may hydrolyze to give a saturated dihydroxy product with the liberation of one atom of active oxygen.

d. Epoxide formation. Compounds containing the ethylene oxide ring are known as monoxides, epoxides or oxido compounds. Fokin (66, 67) proposed that the first step in autoxidation of olefinic compounds was the formation of an ethylene oxide ring wherein one atom of oxygen adds across the double bond. He suggested this after a study of the kinetics of the autoxidation. There is little additional evidence to support this hypothesis regarding an epoxide as the primary product of autoxidation.

By oxidizing linolenic acid with thioglycollic acid as a catalyst, Szent-Gyorgyi (203, 204) measured the oxygen uptake and found that only one atom of oxygen was absorbed for each molecule of acid oxidized. He considered that this left three

possible structures for the oxidation product, namely (a) enol, (b) ketone, and (c) ethylene oxide. Acetylation experiments and treatment with ferric chloride demonstrated the absence of the first two and thus left the third as the choice by Szent-Györgyi["]. He considered presence of ethylene oxide was demonstrated by the fact that the product reduced Tollens reagent, a property of aldehydes and ethylene oxides.

Within experimental limits, he found the iodine number of the oxidized material to be the same as that of the unchanged acid. This, according to Szent-Györgyi["] is as it should be with ethylene oxide which should react as follows:



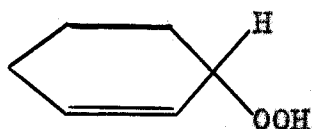
Markley (133) states that the fact that oxido acids of a number of unsaturated acids are well known crystalline products but have in no case been isolated during the early stages of oxidation should be convincing evidence against their formation at this stage of oxidation. Ellis (49) claims to have isolated oxido-elaidic acid as the oxidation product of both elaidic and oleic acids, although he oxidized the acids to a 20 per cent oxygen uptake. Oxido-oleic acid apparently was not formed.

After oxidation of elaidic acid by H_2O_2 in acetic acid, King (114) was able to isolate what he called an oxidostearic acid which was apparently identical with the oxido-elaidic acid of Ellis and had the same melting point (55.5°).

Browne (29) is inclined to the view that one atom of oxygen may add to the double bond with the liberation (from the oxygen molecule) of one atom of nascent or active oxygen which may then attack other fatty acid radicals including saturated radicals. He considers that the action of moisture would cause hydrolysis (after rearrangement to a ketone) to a lower free fatty acid and a lower glycerol ester.

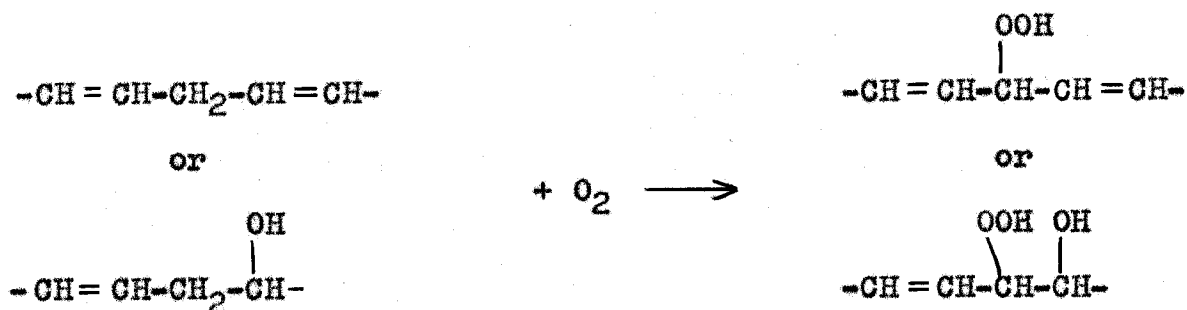
Other workers have proposed oxido compounds as secondary oxidation products, as will be mentioned later.

e. Hydroperoxide formation. Criegee (39) and Criegee et al. (40) suggested that cyclohexene upon autoxidation formed a peroxide with the structure

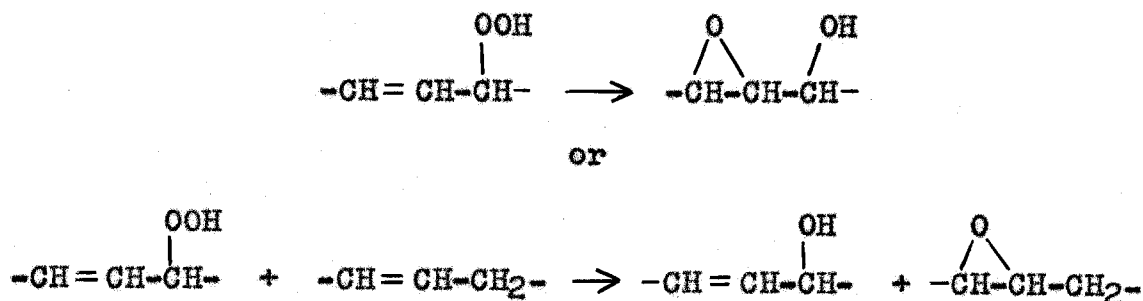


thus leaving the double bond intact. They showed that the peroxide formed (a) was reduced by sodium sulfite to cyclohexen-3-ol, (b) absorbed 1 mole of bromine per mole and (c) contained one atom of active hydrogen per molecule.

Rieche (177) considered that unsaturated fats and oils probably behaved in a similar manner. The suggested mechanism is shown in the following equation.



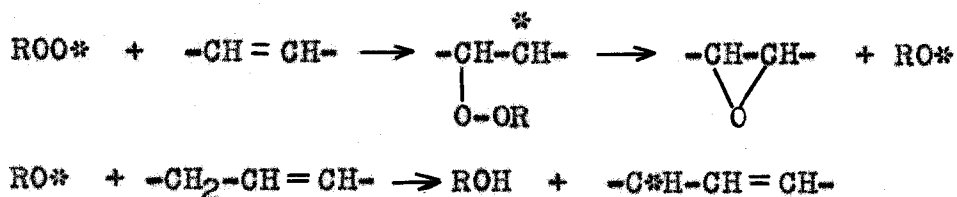
In 1942, Farmer and Sundralingam (59) confirmed the work of Criegee. They isolated a peroxide which behaved in the same manner as described by Criegee, et al; this compound was said to be quantitatively reduced by hydrogen (Adams catalyst) to cyclohexanol rather than to cyclohexane-1,2-diol. The exact figures on hydrogen consumption at this stage are not given; it is stated that two moles of hydrogen were absorbed. A sample of the peroxide was irradiated with ultraviolet light. After the peroxide oxygen content had fallen to about half its original value, the hydrogen uptake was found to be about half that for the original material. This was interpreted to mean that the loss of one hydroperoxide group had resulted in the saturation of one double bond. This could occur in the following ways:



and yield epoxides. The reactions listed above were considered (61) to be the principal secondary reactions although some molecules do reach the chain scission stage. In some cases these secondary reactions begin almost at the outset of autoxidation. With dihydrofarnesene the entire oxygen uptake persists as peroxide oxygen to a 6 per cent uptake while with dihydromyrcene and squalene the decay of peroxide starts almost immediately. Farmer and Sutton make the pertinent statement (61, p. 142) that

. . . an uneven attack (by oxygen) is by no means unusual in the autoxidative reactions of olefinic substances and hence it is probable that many statements in the literature purporting to refer to the early stages of oxidation really refer to fairly advanced stages so far as the molecules actually attacked are concerned.

Alpha-methylenic reactivity was discussed at great length by Farmer and co-workers in 1942 (54, 55, 57). They suggest (57) that interaction of hydroperoxide with a double bond may occur to give epoxy and hydroxy compounds. An alternative suggestion is that since the reaction occurs with greater ease during actual autoxidation, it may occur as a reaction of a radical peroxide with a double bond, as



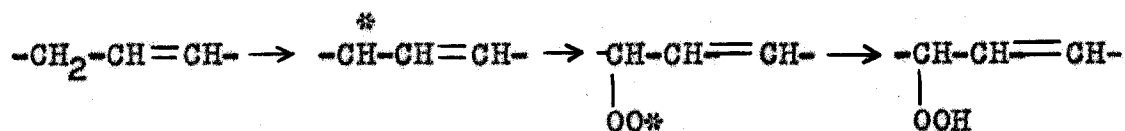
Although their earlier studies were with compounds having smaller molecules, Farmer and Sutton (61) found that their hypothesis of alpha-methylene reactivity applied to the peroxidation of rubber.

As further proof of the validity of their hypothesis Farmer and Sutton (62) isolated the hydroperoxides of photochemically oxidized methyl oleate. The products were mainly monohydroperoxides but some dihydroperoxides were found. The olefinic unsaturation was retained. The monohydroperoxides could be hydrogenated to hydroxystearic acid or reduced with aluminum amalgam to give methyl hydroxyoleate.

In 1946, Swift et al. (201) isolated and characterized a methyl hydroperoxidoleate from oxidized olive oil. While the structure could not be rigorously proven, these authors believed their work supported the views of Farmer and his co-workers. The evidence they presented may be summarized as (a) the iodine value of the oxidation product (71.3) indicates unsaturation (it is lower than the calculated 77.4, probably because of impurity), (b) one mole of peroxide absorbs 2 moles of hydrogen (actually ca. 1.93) to form monohydroxy stearate, (c) the acids resulting from permanganate oxidation are those expected from the hydroperoxide (other compounds might yield the same compounds), (d) the molecular refractivity, 94.7, agrees well with the calculated 95.1 and (e) reduction with HI yields methylhydroxyoleate.

The isolations just mentioned were achieved by molecular distillation and low temperature crystallation. Fugger and his co-workers were able to effect a similar separation by countercurrent extraction (71, 72, 219). Later, Coleman et al. (37) obtained a concentrate containing 70-90 per cent peroxide by complexing with urea.

Farmer and his co-workers (57, 58) believe that olefinic peroxidation occurs by way of a free radical mechanism in which the olefin passes through the stages:



The free radical centers are indicated by asterisks. The first step in the autoxidation of the unconjugated unsaturated oils (and other olefins) is thus the severance of a thermally or photochemically activated alpha-methylene carbon to hydrogen bond leaving an olefinic free radical. Since one may expect resonance between the two three-carbon forms



the addition of a molecule of oxygen would probably result (with equal frequency) in the production of hydroperoxide groups at either the 1 or 3 position.

If the above is correct, double bond shifts should occur in mono-olefinic compounds and oleate should give the

hydroperoxide forms:



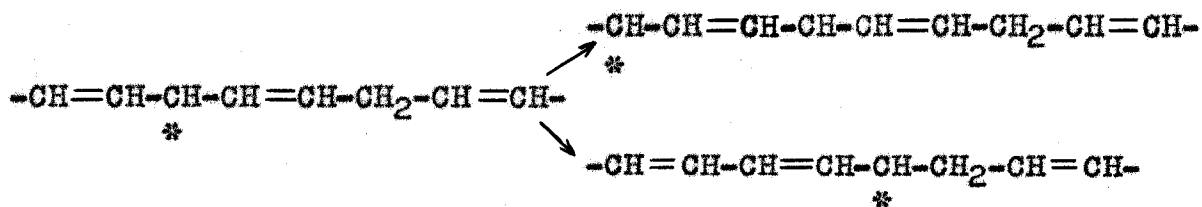
in addition to



These compounds are in accordance with the results of Farmer.

Bickford et al. (13a) worked with the maleic anhydride adduct of methyl oleate and obtained evidence to support the above mechanism. Working with a compound having substituents on the olefinic carbons, Farmer and Sutton (64) were able to show a migration but not saturation of the double bond. The photo-oxidation product of 1,2-dimethylcyclohex-1-ene appeared to contain both 1,2-dimethylcyclohex-1-ene-3-hydroperoxide and 1,2-dimethylcyclohex-2-ene-1-hydroperoxide.

According to Farmer (58) the most reactive methylene groups are those flanked on either side by a double bond. Thus, with linolenate for example, a single attack by oxygen at one of the two more reactive centers could produce the following forms:



By absorption spectra and isolation and characterization of oxidation products, Allen and Kummerow (3) found additional evidence that conjugated and isolated double bonds give different initial products during autoxidation.

Treibs and Rothe (210) believed that in autoxidation of 1,3- and 1,4-multiple unsaturated oxygen active long chain fatty acids, double bonds are saturated with formation of peroxides as primary products while with oleic acid, hydroperoxides are formed with double bonds migrating through the molecule.

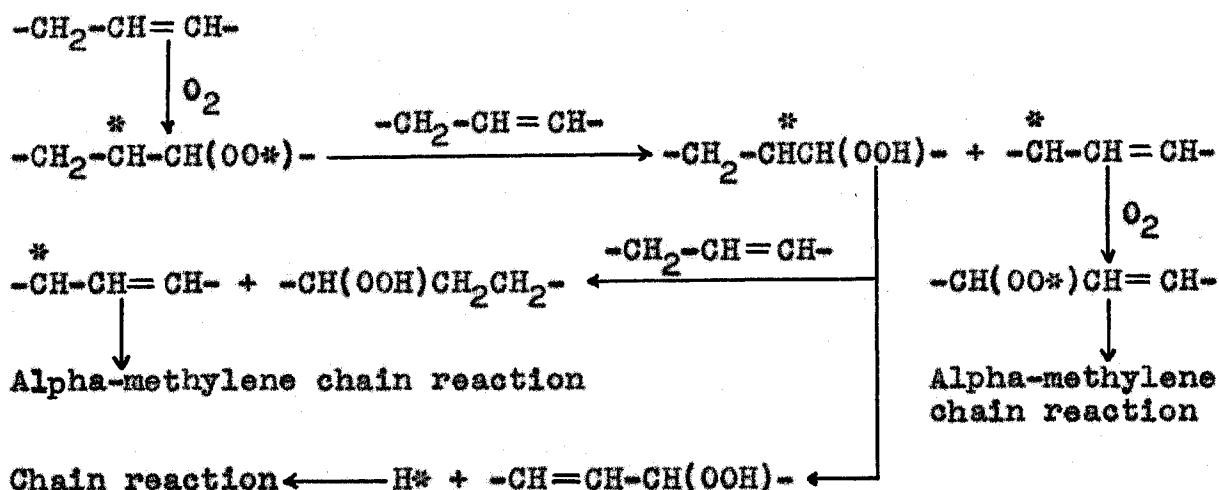
Some confirmation of Farmer's views were obtained by Atherton and Hilditch (6) who found that autoxidation at ordinary temperatures involved conversion of methylene carbon C_8 or C_{11} into hydroperoxide, although some oxidation product must be formed by other means, while at 120° , there seemed to be an almost complete absence of hydroperoxide. The following year Gunstone and Hilditch (84) confirmed this work at 20° , but considered the alpha-methylene reactivity to occur to some extent at 100° to 120° . With methyl linoleate, they found the attack to be almost exclusively at C_{11} and not at C_8 or C_{14} .

Lundberg and Chipault (129) agree with Farmer and Bolland and their co-workers that in the oxidation of methyl linoleate, the initial attack occurs at C_{11} . They, however, postulate the formation of a transitory intermediate which then

undergoes further change. From this intermediate arises a relatively stable 9- or 13-hydroperoxide, but no 11-hydroperoxide of stable character. In a later publication (132) it was indicated that 11-hydroperoxide might also be formed.

Oxidation of methyl elaidate (132) to the extent of ca. 0.2 moles of oxygen per mole of ester showed that 90 per cent or more of the oxygen survived as peroxide. The oxidation of elaidate apparently followed the same course as did methyl oleate.

The detachment of an alpha-methylene hydrogen atom requires about 80 kcal. of energy per mole. The fact that there is no visible source of this energy has been employed as a criticism of Farmer's hypothesis. In reply Farmer (56) states that all investigated conjugated compounds, including those with unsaturated centers flanked by alpha-methylene groups, undergo peroxidation additively at the double bond, leaving the adjacent alpha-methylene group intact. It is, therefore, suggested that possibly oxygen may begin its attack additively in some few olefin molecules thereby furnishing the energy for subsequent chain reactions. This may be illustrated at follows:

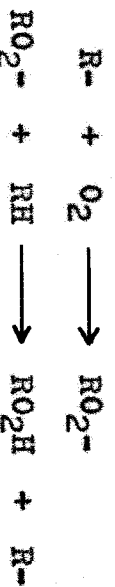


Later Mukherjee (146) also considered the energy aspect of the reaction. He considered that the initial stage of the reaction in the oxidation of oleic and linoleic acids and their esters was cyclic peroxide formation at the double bond. Subsequently more oxygen is used than can be accounted for in this way, probably by formation of hydroperoxides; the energy liberated in the formation of peroxides may be able to remove hydrogen from active methylene groups. This he considers to reconcile the differences between the hydroperoxide and cyclic peroxide hypotheses of fat oxidation.

This brings to mind the words of Gunstone and Hilditch who said (84, p. 840):

. . . the onset of oxidative rancidity in edible fats is largely conditioned by the presence of linoleic rather than oleic glycerides, the peroxides formed by the preferential oxidation of the former catalysing the oxidation of the (usually predominant) oleic glycerides present.

Bolland and his co-workers (16, 17, 18, 21) found their data, obtained in studies of the kinetics, thermochemistry and mechanism of olefin oxidation, to be in agreement with the hypothesis of alpha-methylene reactivity and chain reaction. They represent the chain propagation reactions as



Termination occurred through interaction of pairs of free radicals. In these equations RH represents ethyl linoleate, R·, the radical produced by the removal of an alpha-methylene hydrogen atom from RH and RO₂·, the corresponding peroxide radical.

By allowing equal weights of methyl hydroperoxido oleate and oleic acid to react at 90° for 3 days, Swift and Dolléar (199) found that the iodine number dropped from 69.4 to 66.6, and that 16 per cent of the free acids were epoxy acids, thus showing that even though the epoxy acids may be present, they are not necessarily the first products formed.

Lundberg et al. (132) consider that a Woburn iodine value of 2.12 double bonds per molecule for the oxidation product of methyl linoleate, the peroxide of which had been reduced by potassium iodide, indicates that the double bonds remain undisturbed though possibly shifted during peroxide formation.

In 1949, Dugan et al. (45) found an absorption band (at 3445 cm⁻¹) in the infra red absorption spectrum of autoxidized

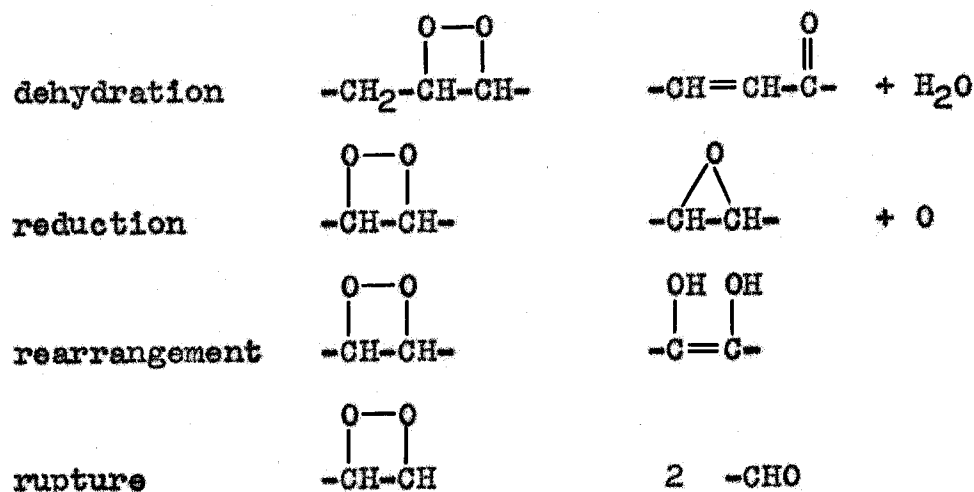
3. Peroxide decomposition products

The type and amount of peroxide decomposition products varies considerably with autoxidation conditions. If temperature changes can alter the type of autoxidation mechanism, they can certainly change the type of materials formed. In the oxidation of cyclohexane and related compounds, Farmer and Sutton (59) considered that if the temperature were allowed to rise above 40° or if peroxide is formed so slowly (e.g., without catalysis by light or other agency) that oxidation is unduly prolonged, the yield of pure peroxide is diminished and that of secondary products increased accordingly. Other substances are said to be similar to cyclohexane in this respect.

Although Fugger and co-workers (71, 72) studied the fatty acid oxidation products by countercurrent distribution methods (and noted that chain scission products were present) they did not examine the scission products in detail.

Water and carbon dioxide are evolved during the autoxidation of fatty acids according to Ellis (48). They are produced in amounts considerably below equimolecular proportions to the acid and are probably a secondary product resulting from a gradual degradation of the fatty acid chain.

Although he expresses his results in terms of cyclic peroxide, Powers (172) lists four types of degradation which may result. These are shown as follows:



Several workers have studied the products resulting from the disruptive permanganate oxidation of the products of autoxidation (when generally the autoxidation was not carried to extreme). While this work was done in an attempt to determine the nature of the primary and the secondary products of oxidation, it does not necessarily follow that these are the same as those formed as a result of autoxidation. The general products formed will be discussed. From methyl oleate autoxidized at 20°, Atherton and Hilditch (6) and Gunstone and Hilditch (84) found azelaic, suberic, octanoic and nonanoic acids. At 120° the products were mainly azelaic and nonanoic with traces of suberic and octanoic acids and possibly some aldehydes. Similar results were obtained by Swift *et al.* (201).

From the autoxidation of elaidic acid, Ellis found (49) the following products: CO₂, H₂O, oxido-elaidic acid, perhaps some oxido-oleic acid and cleavage products. When a 20 per

cent oxygen uptake is recorded, a large portion of this is evolved as CO_2 and H_2O . The former may account for 25 per cent of the oxygen taken up and the latter for even more. Oxido-elaidic acid is formed to the extent of 16 to 20 per cent of the acid autoxidized. With a 20 per cent oxygen uptake the 120 parts of product from oxidation of 100 parts elaidic acid included 16 to 20 parts azelaic, suberic, oxalic, nonanoic and octanoic acids.

Farmer and Sutton (61) in 1942 reported that in their experience all polyisoprenes, and notably rubber appear to give neutral scission products initially and only later are these converted to acid products. They say that doubtless these neutral products terminate in aldehydic and ketonic groups; the former can probably absorb oxygen directly to form peracids and then carboxylic groups, but the hydroperoxides probably play a large part in the further oxidation:



The following year, Farmer and Sundralingam (60) noted that the products resulting from the oxidation of rubber were generally neutral (insoluble in aqueous alkali) up to a utilization of 0.9 atom of oxygen per isoprene unit; beyond this the oxidation products became increasingly acidic until at the highest stage of oxidation (30 per cent oxygen input at 70°) only acidic materials were obtained. Apparently the

first units produced have neutral end groups such as $-\text{CHO}$ and $-\text{COCH}_3$; these end groups in the main escape further oxidation until a fairly definite overall stage of oxidation is reached.

It had been stated earlier by Farmer and Sundralingam (59) that oxidation of olefinic compounds generally results in the formation of a compound with a hydroxyl group and this is often accompanied by a ketone. They feel that it might seem at first sight (particularly in view of the fact that water is formed) that direct dehydration of olefinic hydroperoxide might have occurred. However, they were unable to demonstrate any cyclic ketone in the autoxidation of cyclohexane.

They further state (39) that hydroperoxide groups decompose spontaneously or by the action of heat, giving (a) cyclic epoxides and alcohols, (b) oxidation products and (c) oxygenated polymeric products. Ketones are not formed in recognizable amounts by the spontaneous decomposition of the cyclic hydroperoxides although they have long been known to appear among the autoxidation products of mono-olefinic terpenes; they are formed, however, to a considerable extent by the violent decomposition promoted by an organic ferrous salt.

In a theoretical discussion, Gibson (74) considered that the chief decomposition products of the hydroperoxides would be α -unsaturated hydroxy esters; α -, β -dihydroxy

esters, and carbonyl compounds.

Bolland and Koch (19) considered, on the basis of absorption at 2750 Å, that one of the decomposition products of the peroxide of ethyl linoleate was a conjugated diene ketone (which actually has triene conjugation).

Dugan et al. (45), by infrared spectrographic analysis, considered that two different carbonyl compounds are formed (at different rates) in the autoxidation of methyl linoleate. One is believed to be a ketone in conjugation with a double bond while the other is believed in a nonconjugated system. In a similar study on the methyl esters of peanut oil fatty acids, Lemon (126) found that in the C=O stretching region (1840-1630 cm^{-1}) three bands appeared at different stages of autoxidation.

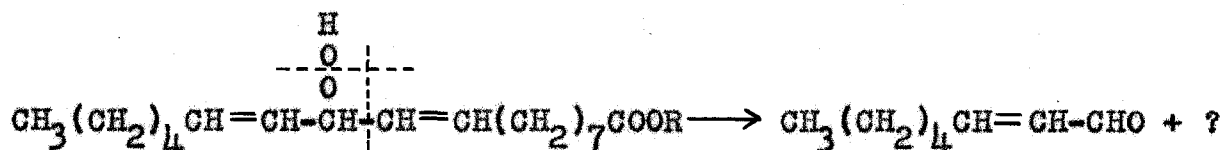
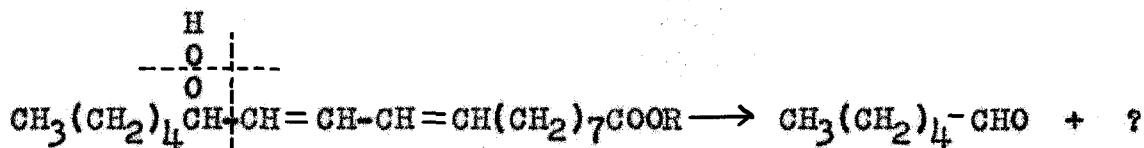
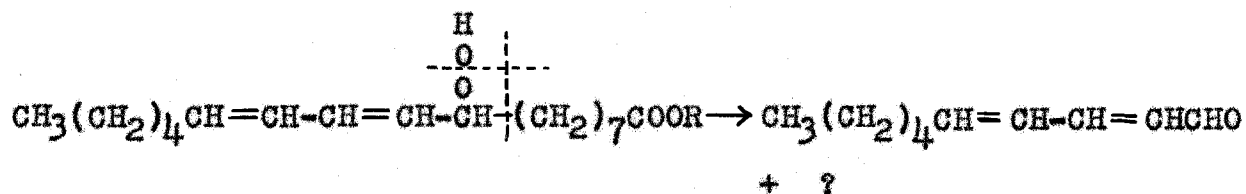
At various temperatures from 50° to 100° Ellis (50) found alpha, beta-unsaturated keto acids (11-keto- Δ^9 - and 8-keto- Δ^9 -oleic or elaidic acids) or their polymers. Small amounts of beta-ketonic acids were noted. Autoxidation at 100° yielded eight and nine carbon mono- and di-carboxylic acids.

In a study of the decomposition (by heat at 150°) of methyl hydroperoxidooleate, Swift et al. (200) were able to demonstrate the presence of 2-undecenal. They state that

Qualitative evidence has been obtained in the course of the isolation and purification of 2-undecenal of the presence in the decomposition mixture of analogs and homologs of this aldehyde. Aldehydes of the type represented by 2-undecenal

readily undergo autoxidation, reduction, and isomerization thus making it possible to account for many of the end products which have been qualitatively and quantitatively identified in various types of fat oxidizing systems.

In a later paper (202) they reported the finding of dec-2,4-dienal, oct-2-enal and hexanal in autoxidized cottonseed oil. Although the exact mechanism of their formation was not established it was believed that they resulted from decomposition of isomeric hydroperoxide of linoleic acid. The following equations suggest possible pathways.



From oxidized milk fat, Keeney and Doan (110, 111, 112) were able to distill a volatile fraction having odors characteristic of the fat. The greater part of this fraction was shown to consist of carbonyl compounds, particularly ketones. While the ketone and the non-carbonyl neutral fractions each, when mixed singly with milk, gave a flavor suggestive of oxidized flavor, the flavor most nearly resembling

that of true oxidized flavor was obtained by adding to the same milk 0.05 ppm of the former and 0.5 ppm of the latter. The ketone fraction was shown to consist of several ketones, none of which were definitely identified, though it was shown that none were methyl ketone and most were unsaturated. Aldehydes were not recovered in the procedure used, but may play a part in the oxidation processes.

4. Relationship to season of year

Mattick (134a) discusses the defect of oxidized flavor under the name of "oiliness in milk". He noted that dairies in England found the flavor complaint much more widespread in the winter months. The flavor could be induced by copper if the milk was held cold (i.e., below 10°) but not if held at temperatures approximating summer temperatures.

While he gave no data on this subject, Anderson (5) mentioned that in winter months, many milk dealers have disturbing experiences with oxidized flavors.

Trout and Gjessing (211) state that "the high stability of summer milk against oxidized flavor development, even with copper contamination within the plant, is well recognized commercially." They further noted that grade A raw milk was occasionally criticized for this flavor in late fall and winter but it was not noted in the summer months of their study. With irradiated pasteurized milk, the flavor was noted

more frequently in fall and winter, while with the regular pasteurized milk in their study, the flavor did not develop even in winter.

Although Webb and Hileman (215) considered summer milk to be less susceptible, it should be pointed out that during the first winter they studied the problem and the following summer, the milk was exposed to copper. The following winter, without copper contamination, there was apparently less oxidized flavor. However, these workers seem to have used very small numbers of samples. In fact, during the second winter, they state that 14 per cent of the samples became oxidized; this actually represented only one oxidized sample.

After a study of the cow as a source of oxidized flavors in milk, Guthrie and Brueckner concluded that oxidized flavors were more pronounced and more widespread in winter than in summer (30, 86). While it is believed that the majority of people who have worked closely with the market milk industry would likely agree with this (at least as applied to mixed herd milks) the data presented by these workers for individual cows does not seem entirely conclusive on this point.

Since the oxidized flavor usually disappears in the summer when the cows are on pasture, Dahle (42) believes that the feed may have some effect. According to Greenbank (77) "Inhibition of the (oxidized) flavor by a change from dry to green feed is paralleled by a decrease in the

oxidation-reduction potential and an increase in poisoning action".

Brown, Thurston and Dustman (28) found the mixed milk of several cows more susceptible to copper or iron induced oxidized flavor in winter than in summer. Feeling that the difference was due to the change from pasture to dry feed, they studied reversals between dry- and pasture-feeding regimes, the study showing some tendency for greater susceptibility on dry feed. Thurston (401) further noted that in some summers the flavor occurred coincidental with drought conditions which necessitated considerable amounts of dry feeding.

5. Influence of external factors

a. Influence of air and oxygen. It has been found by several investigators (31, 152, 192) that autoxidation does not proceed in the absence of air or oxygen. Burger and Wiedemann found (31) that no appreciable amounts of conjugated unsaturated acids were formed from esters of fatty acids in the absence of air, while Skellon (192) noted that even in the presence of a limited amount of air a small amount of peroxidation occurs.

Mukherjee (152) studied the influence of oxygen concentration, ranging from one per cent to 100 per cent. Decreasing the oxygen content of the storage atmosphere was almost without effect on the induction period, while subsequent oxidation was greater in air. Increasing the oxygen concentration

from 21 per cent to 100 per cent has no effect on the length of the induction period until the atmosphere is pure oxygen, but the rate of subsequent oxidation is greater. Dissolved oxygen was determined by adsorption in hyposulfite or alkaline pyrogallol solution and it was found that increased peroxide formation was accompanied by an almost proportionate increase in dissolved oxygen. When all the oxygen is used up, peroxidation stops and is followed by decomposition of peroxides. Autoxidation is thus said to depend on the rate of diffusion of oxygen from the atmosphere to the oil, and is controlled by the solubility of the gas in the fat or oil concerned.

Some mention has already been made in connection with a review of the literature pertaining to reaction mechanisms of the amount of oxygen different investigators found to be taken up by various olefins.

Meyer (136) found that in the hemin catalysed photo-oxidation of methyl oleate, the reaction slowed down considerably after the absorption of only one atom of oxygen per molecule of ester, while the work of Treibs (209) showed that in the autoxidation of both methyl linoleate and methyl linolenate, two molecules of oxygen were absorbed.

Ellis (48, 49) measured the oxygen uptake by oleic and elaidic acids under conditions of high dispersion. In 24 to 48 hours, either acid was able to take up over 20 per cent of

oxygen (or more than 56.5 grams per mole) at 55 to 75°. He observed that under the most favorable conditions, elaidic acid absorbed three to four atoms of oxygen and that two or three of these were retained in non-volatile oxidation products.

The rate of oxygen uptake by oleic, ricinoleic, linoleic and linolenic acids was investigated by Franke and Jerchel (69). At 37°, even with one per cent $\text{Co}(\text{NO}_3)_3$ as a catalyst, oxygen absorption was in no case complete after 100 hours. With oleic and ricinoleic acids the rather rapid initial absorption slowed down after 0.3 to 0.5 mole oxygen were absorbed per molecule of ester and was still less than one mole after 100 hours. After the absorption of one mole of oxygen by linoleic acid and two moles of oxygen by linolenic acid, the curves tended to resemble those for oleic and ricinoleic acids. There was apparently some tendency for the polyunsaturated acids to slightly exceed one mole of oxygen per double bond.

In a study of oxygen absorption by methyl linoleate, Lundberg and Chipault (128, 129) noted that in the early stages of peroxide development, the determined peroxide values were always higher than the theoretical values on the basis of oxygen absorption. This was attributed to a decrease in amount of dissolved oxygen. After correcting for this, a straight line relationship was found between measured peroxide

values and oxygen absorption to 300 milli-equivalents peroxide per kilogram of ester. The curves plotted did not coincide with the theoretical line, which may result in part (but apparently not entirely) from peroxide decomposition. Measurements were made at 40°, 60°, 80°, and 100°; the higher the temperature the greater the deviation.

With a sample oxidized in a closed system at 30° Allen et al. (2) were able to demonstrate that up to a point of 0.2 mole oxygen absorption, all oxygen taken up could be demonstrated as peroxide oxygen.

b. Effect of light. While most workers in the field of fat oxidation seem to feel that light (especially ultra violet light) catalyzes the oxidation of fats and other olefinic materials, little work seems to have been done to establish a definite relationship between light and oxidation.

Skellon (192) stated that ultraviolet light acts rapidly on purified oleic acid held in an open vessel in a slow stream of oxygen. Farmer et al. (57) state that absorption of oxygen is promoted by sunlight or ultraviolet light; decomposition of hydroperoxide is apparently promoted by prolonged illumination.

Lea (121) noted almost no oxidation in absence of light; he concluded that a small amount of light may exert a powerful influence on the rate of oxidation. Increasing the amount of light beyond this small quantity did not greatly increase the

rate of oxidation. Removal of light after oxidation had started did not have much affect in decreasing the rate.

In a study of oxidation in the presence of chlorophyll, Lundberg and Chipault (129) noted that in the absence of light, the uptake of oxygen was almost too small to be measured while with light, the effect was very pronounced. Oxygen absorption was found to be proportional to the amount of light absorbed. The conjugated diene peroxides formed were considered the same as those produced under other conditions of oxidation. The results were, therefore, considered to indicate that the photo-oxidation of fats depends upon the activation of pigments or chromophores that are present as minor constituents. These same authors and their co-workers in later papers (129, 131) concluded that the fundamental mechanisms for thermal and photochemical oxidations were quite different. In 1942, Farmer et al. (57) had concluded that it was impossible at that time to distinguish between thermal and photochemical secondary decompositions.

Rogers and Taylor (181) concluded that oxidation was not proportional to illumination intensity after studying rate of absorption of oxygen by linseed oil under influence of light. Oxidation was negligible in the absence of light; the weaker the light, the greater was its efficiency in catalysing oxidation.

According to Mukherjee (149), Lea (121) attributed the fact that apparently sweet fats become (oxidatively) rancid when exposed to light (out of contact with air) to the presence in the fat of minute amounts of oxygen, either in solution or in loose chemical combination. While such reasoning seems quite logical, no such remark can be found in the paper to which reference was made.

Mukherjee (147, 149, 153) finds light to be a positive catalyst for oxidation. Samples stored in diffused daylight in absence of oxygen did not show organoleptic evidence of oxidation. In a study of the relationship between amount of illumination and oxidation, he found (149) that if samples were illuminated prior to storage, there was a relationship between the duration of exposure and the rate of peroxide development. Once a fat has been exposed to light rays to start oxidation at a sufficient rate, the process cannot be stopped by removal of the exciting source. However, light is only one factor in oxidation, since oxygen is considered to be necessary.

c. Effect of temperature. The present study was made using 40° as the highest storage temperature. Most of the available literature on the effect of temperature deals with studies wherein the lowest temperature observed was 40° or higher. Especially in a material such as milk fat, this might be particularly important by reason of the fact that at 40°

the fat is liquid while at the other temperatures in this study, the fat is not entirely liquid. Nevertheless, some of the literature on the subject of temperature will be very briefly reviewed.

Several workers have suggested that there may be a different mechanism of oxidation at higher temperatures. After studying the acid scission products from disruptive perorganate oxidation of the autoxidation products of methyl oleate, Atherton and Hilditch (6) concluded that at 20° oxidation was largely at alpha methylene groups, while at 120° the action was mainly at the ethenoid bond. From a study of rate of peroxide development, Gunstone and Hilditch (84) considered the oxidation at 80° and above to differ from that at 50° and below. Fuell and Skellon (65) seemed to feel that the mechanisms at 55° and 85° were similar, but considered that at 120° to be different. Hess and O'Hare (95) studied the range 80°-200° with linseed oil; they found that in the range 80°-130°, the length of the induction period increases logarithmically as temperature is reduced, while above this range the induction period is practically negligible. In their study of oxidizing methyl linoleate, nothing was said by Lundberg and Chipault (128, 130) as to whether an induction period was noted at 80° and 100°. At 40° and 60° non-reproducible induction periods varying from a few minutes to five or six hours were noted. It might be well to note that this and possibly

other studies were made while the sample was being agitated.

While Lundberg and Chipault (128, 130) noted that the rate of oxygen absorption increased approximately twofold for each 20° rise in temperature (40°-100° range), they consider that this temperature coefficient has no theoretical significance, but is dependent upon experimental conditions. These results are slightly at variance with those of Bolland and Gee (17). They (17) present a graph (but no figures) which shows that autoxidation as measured by rate of oxygen uptake of ethyl linoleate at 55° is about twice that at 45° and four times that at 35°.

Gunstone and Hilditch (84) list the times for methyl oleate to reach a peroxide value of 500 milliequivalents per kilogram as follows: about 600 hours (20°), about 380 hours (50°), 37 hours (80°), 9 hours (100°) and 2.5 hours (120°). They found temperature coefficient with methyl linoleate to be more constant where the times to reach a peroxide value of 1500 were 121 hours (20°), 19 hours (50°) and 4 hours (80°).

Mukherjee studied effects of temperature (148) on oxidation of butterfat, but the lowest temperature he used was 60°. He did find, however, that the decomposition of the peroxide increased progressively as temperature increased. In their study of the isolated methyl hydroperoxido oleate, Swift *et al.* (201) found that storage at 0° for one month gave no discernible change. Upon storage in an open beaker exposed to daylight,

(25-28°) the peroxide content decreased 13 per cent and 30 per cent in 30 and 60 days respectively. At 120° and above, decomposition was very rapid, with fifteen minutes at 150° being sufficient for complete decomposition. Paschke and Wheeler (167) had earlier found that the speed of decomposition of peroxide becomes progressively greater as the degree of oxidation is increased, the rate of decomposition generally conforming most nearly with that of a bimolecular reaction.

Paschke and Wheeler (167) assumed that one molecule of oxygen saturates one double bond to form one molecule of peroxide and developed an equation for theoretical peroxide formation based on decrease in iodine value. Their results showed that observed and theoretical peroxide values for methyl esters of soybean fatty acids (theoretical maximum 9000 meq. per kg.) coincided only to about 500-600 at 100°. Maximum values were obtained at 75°, at which temperature the values were 1000 (observed) and 1700 (theoretical). At 55° the values were 1700 and 2400 respectively while at both 35° and 15° the figures were 2600 and 3100.

Calculating a theoretical peroxide value based on oxygen absorption, Lundberg and Chipault (128, 130) noted that the observed values plotted against oxygen uptake were straight lines (to 300 meq. per kg.) at 40, 60, 80, and 100°, but that at all temperatures, the observed values were less than the theoretical maximum. The deviations increased with increasing

temperature. They consider that this is not all due to peroxide decomposition since at 40° , peroxide decomposition is considered to be very slow.

An anomalous result was mentioned by Banks (20) who stated that the fat of dehydrated herring was more stable when the herring was stored at 25° than when stored at 10° . No explanation for this is given.

According to Ellis (48) stearic acid shows appreciable autoxidation above 75° .

6. Oxidation catalysts

Various metals have long been recognized as being able to promote the development of an oxidized flavor in dairy products, the most common offenders noted being copper and iron. Both Sommer (194) and Roadhouse and Henderson (178) recognize this in their texts on market milk. Nelson and Trout in their book (163) on the judging of dairy products not only recognize this, but prescribe a method whereby, through the addition of copper, the susceptibility of various milks toward the development of oxidized flavor may be determined.

The addition of 0.01 and 0.001 mg. of copper (as stearate) per ml. of autoxidizing methyl linoleate was found by Lundberg and Chipault (129) to virtually eliminate the induction period at 40° . They reported a more detailed study in a

later paper (131). A study of spectral absorption at 2325 Å in relation to peroxide content in oxidizing methyl oleate showed that a larger proportion of chromophores is formed in the presence of copper than in its absence. The presence of copper produces an increase in the number of chains (but a decrease in chain length) and the chromophores are produced in chain termination reactions. The conclusion is reached that the prooxidant effect of copper is due to its effect on peroxide destruction rather than to a direct catalysis of the reaction between oxygen and linoleate; the decomposition of peroxide forms free radicals which initiate new oxidizing chains.

It was reported by Lemon et al. (126) that while increased temperature accelerated both formation and decomposition of peroxides, the presence of ferrous stearate catalyzed only their decomposition.

The presence of about 0.1 per cent of the cobaltous salt of oleic or elaidic acid was found by Ellis (48) to be sufficient to reduce or completely eliminate an otherwise more or less prolonged induction period. In a later study (49) he used 0.2 per cent (and other changed conditions) to speed up autoxidation.

The oxidation of oleic acid was studied at 120° in the presence of various metal catalysts by Skellon (190, 191, 192). The catalytic activity of the metal ions was correlated with

their structure and shown to be a periodic function. High activity was shown by metals having an incomplete inner group of electrons. An element of dual valency, attaining a stable condition in the higher valency should be an efficient catalyst. Autoxidation is considered to function through a cycle of valence changes as follows: (a) the formation of a loose complex between metal in the lower valency and oxygen, the metal being raised to a higher valency; (b) this higher valency is more stable, but the complex is still highly reactive to oxygen, reacting further with oxygen and the oxidizable substance, donating oxygen continuously to the latter without permanently reverting to its lower valency; (c) ultimate interaction between the catalyst in its higher valency and a quantum of oxidized material causing reversion of the metal to its lower valence, accompanied by a change in the character of the oxidized material.

Mukherjee (151) studied the actions in butterfat of several metals in metallic form, as water soluble salts and as oleates. In all three cases, copper and iron were the strongest prooxidants. Copper and cobalt were studied in the presence and absence of oxygen and were unable to promote oxidation in absence of oxygen.

In a study of the catalytic effect of oxidation products of butterfat, Mukherjee also found (150) that the peroxides were largely responsible for the prooxidant effect and apparent

autocatalytic nature of oxidation, while the materials volatile at 100° (having a strong aldehyde reaction and including products responsible for the odor of oxidized fat) are inactive as catalysts.

7. Antioxidants

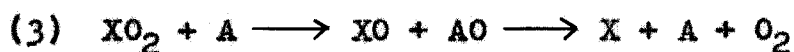
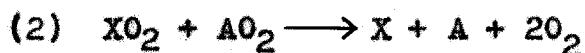
It has long been recognized by most workers in the field of fat oxidation that most natural fats and oils exhibit an initial period, the induction period, when oxidation as measured by any known standard, is extremely (sometimes immeasurably) slow. The length of this induction period is apparently dependent upon some naturally occurring substances in the fat or oil (11, 97, 122, 128).

In 1932, Hilditch and co-workers considered that the most efficient "antioxygens" found by that time were hydroxylic compounds such as quinol and pyrogallol, although their study dealt with substances accompanying natural fats (11, 97). While they were unable to identify the antioxygens, they found that extraction with boiling water destroyed them and in some cases boiling water with 8 per cent aqueous HCl was even more effective. With olive oil, about 0.03 per cent quinol restored a resistance to oxygen uptake of about the same order as that originally present.

It has been shown by a large number of workers (for example 4, 15, 21, 81, 105, 125, 129, 153, 154, 155, 205) that

the addition of various substances, notably phenolic materials, can either greatly delay the onset of active oxidation or slow down the rate of oxidation.

Much of the early work regarding the mechanism of action of antioxidants is based on the work of Moureu and Dufraisse (145) who explained the action according to one of the three following methods:



where A represents antioxidant and X the oxidizable substrate. The explanation for the end of the induction period has been that it represents the time when essentially all the antioxidant has been oxidized (or otherwise changed) so as to become ineffective. However, the above mechanism does not seem to fit as well as it might with the well substantiated chain reaction mechanism of autoxidation.

In 1947 Bolland and ten Have (21) studied the kinetics of oxidation of ethyl linoleate in presence of hydroquinone and concluded that the hydroquinone interferes with the oxidation in only one way, namely by interaction with peroxide radicals, thereby breaking the reaction chain. While they were unable to identify the product, kinetic evidence was presented indicating that the hydroquinone undergoes a chemical change during this termination reaction.

Before the chain reaction mechanism of autoxidation had become established, Alyea and Backström["] had studied the inhibitive action of alcohols on the oxidation of sodium sulfite and concluded (4) that the action consisted of breaking reaction chains in both thermal and photochemical oxidations. Their evidence showed that in the induced oxidation of the alcohol, two molecules of oxidation product (aldehyde or ketone) are formed for each chain broken. They also found that while at low inhibitor concentration, the amount of alcohol oxidized increased with increasing concentration, at high concentration the amount of alcohol oxidized per unit of time is constant and independent of concentration.

Of the various materials which have been definitely shown to have antioxidative properties, only ascorbic acid and the tocopherols are known to be naturally present in dairy products. The former is a water soluble substance and while present in milk in its natural state, it would not be expected to be present in dry milk fat. This, then, leaves the tocopherols as the known natural antioxidants in dry milk fat.

B. Methods of Studying Autoxidation and Their Application

1. Tocopherols

a. Application to study of milk fat oxidation. As has just been mentioned, the tocopherols seem to be the only naturally occurring antioxidant which one might normally expect to be present in dry milk fat.

Several workers (129, 206, 214, and others to be mentioned later) have noted an antioxidant activity of tocopherol. Lundberg and Chipault (129) while noting that alpha-tocopherol considerably lengthened the induction period, found that the oxygen uptake during this lengthened period was greater than during the induction period without tocopherol.

On the other hand, Reinart (176) felt that the natural antioxidants in butterfat did not appear to influence the stability of butterfat against oxidation. He found (175) that while the vitamin E (alpha-tocopherol) content of butterfat is two to two and a half times higher in late summer and autumn than in winter and spring, the faults in butter from oxidation are highest in summer butter and lowest in winter.

With lard as a test medium, Griewahn and Daubert (83) tested the relative effectiveness of alpha-, beta-, gamma-, and delta-tocopherols as antioxidants; the test was made at 100°. They found the protective action to be as shown in

Table 1, with the value for the alpha form taken as 1.

On the other hand, Kunkel (118) noted in the photooxidation of methyl linoleate at 25°, alpha-tocopherol had a greater antioxygenic activity than did the gamma form.

Table 1
Relative Protective Action of Tocopherols^a

| Lard | Amount of tocopherol | Protective action for forms | | | |
|------|----------------------|-----------------------------|---------|----------|----------|
| | | α | β | γ | δ |
| A | 0.02% | 1 | 1.2 | 1.3 | 1.5 |
| A | 0.0% | 1 | | 1.2 | |
| B | 0.1% | 1 | 1.3 | 2.0 | 2.5 |

^aConstructed from the data of Griewahn and Daubert (83).

Some of this difference may possibly be explainable on the basis of temperature. Hove and Hove (105) studied the antioxygenic activities of the various tocopherols in terms of 50 per cent destruction of carotene and in terms of an increase in ethyl oleate of 20 meq. peroxide per kg. Their results are tabulated in Table 2. Thus it may be seen that while the actual antioxygenic activity decreases with increase in temperature, the decrease is less for the gamma form than for the alpha. Results presented by the authors to 98° show this even more strikingly.

Table 2
Antioxidant Activities^a

| Antioxidant | Activity measured on | | | | | | | |
|------------------|----------------------|-----------------|--------|------|----------------------|------|------|-----------------|
| | Carotene | | | | Aerated ethyl oleate | | | |
| | 4 ^o | 25 ^o | | | 35 ^o | | | 35 ^o |
| | K ^b | R ^c | K | R | K | R | K | R |
| Alpha-tocopherol | 0.095 | 1. | 0.0581 | 1. | 0.0500 | 1. | 18.2 | 1. |
| Beta-tocopherol | 0.084 | 0.88 | 0.0523 | 0.90 | 0.0516 | 1.03 | 14.4 | 0.79 |
| Gamma-tocopherol | 0.098 | 1.03 | 0.0685 | 1.17 | 0.0650 | 1.30 | 17.0 | 0.94 |
| Gossypol | | | | | | | 23.7 | 1.30 |

^aTaken from tables by Hove and Hove (105, pp. 625, 630).

^bActivity of antioxidant.

^cRelative activity of antioxidant with alpha-tocopherol activity as unity.

Mukherjee and co-workers (153, 155) studied various compounds as antioxidants in butterfat. Ascorbic acid was found to be effective, but not to nearly as great an extent as alpha-tocopherol, while a combination of the two was much more effective than either alone. In the other study (155) ethyl gallate, ascorbyl esters and hydrogen were studied. The greatest protection was obtained with a mixture of alpha-tocopherol (0.005 per cent) and ascorbyl palmitate (0.01 per cent). Kesterson and McDuff (113) found alpha-tocopherol more effective in orange oil (at 0.1 per cent) than ascorbic acid,

nordihydroguaiaretic acid, citric acid and several proprietary formulations. In contrast to this, Lehmann and Watts (125) found the following order of decreasing effectiveness: nordihydroguaiaretic acid, butylated hydroxyanisole, propyl gallate, dodecyl gallate and alpha-tocopherol. This agrees with Blaizot and Curvier (15) who found nordihydroguaiaretic acid more effective in lard than either hydroquinone or alpha-tocopherol.

Singleton (189) separated antioxidant fractions from hydrogenated cottonseed oil by means of molecular distillation. The mixtures of tocopherols in the most potent fractions were more effective than alpha-tocopherol alone, but less effective than gamma-tocopherol.

b. Methods. No extensive review of the methods for the determination of tocopherols will be made here. Harris and Kujawski (88) compiled an annotated bibliography of vitamin E covering the period of 1940 to 1950. They have 90 entries in the section on determination. Alpha-tocopherol is generally considered as vitamin E although the other forms do have some vitamin activity. Working in this laboratory, Handwerk (87) made a detailed study of methods for the determination of tocopherol. The method he recommends is an adaptation of the Kjolhede modification of the Emmerie and Engel method. This involves the saponification of the fat and the determination of tocopherol on the nonsaponifiable fraction after removal of

interferents by adsorption of these onto Floridin. The color development is based upon the reduction of ferric iron and the subsequent formation of a color complex between ferrous iron and alpha, alpha'-dipyridyl.

2. Peroxide content

a. Application to a study of milk fat oxidation. Since peroxides are considered to be the first product of the action of oxygen upon an oxidizable material such as a fat or an oil, many workers have felt that if one wished the earliest possible chemical indication of the degree of oxidation, the peroxide content would be the thing to measure.

Gunstone and Hilditch (84) noted that the induction period in methyl oleate oxidized at various temperatures was followed by a much more rapid increase in the peroxide value to a maximum, followed by an equally rapid fall in peroxide value. A similar result was noted by others (95, 192).

In a study of a sample of fish oil acids exposed to air in diffused daylight, Farmer and Sutton (63) found that 1.2 atoms of oxygen were absorbed in seven days, all of which within the limits of accuracy of the method employed, were shown to be peroxides. Although differing slightly from the theoretical curve, a straight line relationship between oxygen absorption and peroxide value was found by Lundberg and Chipault (128) to exist to a peroxide value of 300 meq. per

kg. of methyl linoleate.

At 30°, Allen, Jackson and Kummerow (2) found that up to a point of 0.2 molecules oxygen uptake, all oxygen could be demonstrated as peroxide.

Nelson and Dahle (162) found that fat freshly isolated from milk or cream usually had a peroxide number of zero even from milk or cream having a strong oxidized flavor which carried over into the pure fat. They considered that the peroxide value did not change until the color of the fat was affected, which was some time after the development of flavor defects.

According to Pyenson and Tracy (174), although peroxide formation in all samples was higher after twelve months of storage, the values were not sufficiently correlated with flavor scores to justify the use of the peroxide test as a means of measuring or predicting the keeping quality of whole milk powder. They consider that organoleptic tests remain the most accurate method of measuring changes that occur in flavor and odor of powdered whole milk during storage at either 35° F. or 100° F.

Pyenson and Tracy quote Holm (100) as follows:

The rate of oxidation of fats may be measured by that of formation of peroxides only if the oxidation takes place at relatively low temperatures and if the peroxide values are relatively small.

At higher temperatures the rate of peroxide formation may not be a measure of the rate of

oxygen absorption and the extent of peroxide formation may not necessarily be a measure of deterioration. This is especially true in the case of fats and oils which contain acids of high degree of unsaturation.

The most satisfactory and practical method of measuring deterioration is by the time of appearance of off-odors and off-flavors, which are caused by the breakdown of peroxides.

However, as reported in a later paper (82), Greenbank and co-workers (including Holm) found that the tests of samples packed in air and stored at 20°, 30°, 37°, 45°, and 55° indicate that the rate of peroxide development at temperatures up to 45° can be relied upon as a fairly accurate measure of the rate of autoxidation and as a measure of the relative keeping quality of dried milks, provided the criterion is a relatively low peroxide value. The rate of peroxide development was not, however, necessarily an index of the time of storage required to develop tallowy odors and flavors in a gas packed sample.

In a study of seasonal variations in butter, Mattson et al. found (134b) that butter with a high content of di- and trienoic acids showed a higher peroxide value after storage than that with a low content. The correlation between peroxide value and oily flavor was fairly distinct ($+0.71 \pm 0.05$).

Despite this relation of peroxide value to both oily flavor and concentration of di- and tri-enoic acids, the connection between the latter two was small, indicating that there are other factors influencing the incidence of the oily flavor.

Naumann et al. (161) found that flavor, odor and peroxide values were not closely associated with each other in the development of oxidative rancidity of frozen ground pork, but that flavor was more closely associated with peroxide value than odor.

Of several tests (peroxide value, Kreis value, aldehyde value, acid value of aqueous extract, oxidation-reduction potential, iodine number, melting point, refractive index, fat content, and moisture and volatile content) employed for estimation of rancidity of bacon fat, White (217) considered the peroxide oxygen (iodometric), Kreis, and aldehyde tests to be the most suitable.

b. Methods of determination. For many years the methods for determining the peroxide content of organic materials were based upon the oxidation of iodides and titration (usually by thiosulfate) of the resulting iodine.

Perhaps the most widely used method was that of Lea (122). Powdered KI is added to a weighed sample of fat, dissolved in a mixture of acetic acid and either chloroform or carbon tetrachloride. The solution is flushed with nitrogen or carbon dioxide, is heated to boiling, is cooled and the mixture is titrated with 0.002 N thiosulfate, using starch as an indicator. With a one gram sample, one milliliter of 0.002 N thiosulfate is equivalent to 1 millimole or 2 milliequivalents of peroxide, 16 mg. of active oxygen or 32 mg. of total

peroxide oxygen per kilogram of fat.

There are various modifications of the above. For example, Franke and Jerchel (69) and Goldschmidt and Freudenberg (75) use hydriodic acid instead of an iodide salt; Wheeler (216) uses a larger sample size. There are various other modifications, but these are all based on the principle of oxidation of iodide to iodine.

Stansby (196) considered that many of the tests were quite empirical and after a study of factors affecting the results, suggested some modifications to the Wheeler method, such as reducing solvent from 50 milliliters to ten, and allowing reaction mixture to stand one minute rather than being agitated. He also suggested that the addition of 50 ml. 0.1 N HCl instead of water at the end of the reaction would cause the end point to be easier to attain by rapidly breaking the emulsion.

Various workers noted that with the iodometric procedures, the value increased as the sample size decreased. Lea (124) was able to demonstrate that this was due to dissolved oxygen. He then developed two alternative procedures in which he considered the oxygen error to be eliminated. One was a "cold" procedure in which the sample stood at laboratory temperatures for one hour instead of its being heated to the boiling point of the solvent for two minutes.

Considering that the so called "oxygen error" in iodometric procedures was related to the solvent, Hartman and White (89) developed a solvent mixture which they thought gave a low error and therefore, more accurate results. They felt that their solvent eliminated the need for blanks, a fact which is of particular importance in low peroxide samples. The exclusion of air during the determination was emphasized as essential in any determination.

In 1943, Chapman and McFarlane (35) adapted a colorimetric method to the determination of peroxide in dry milk. The method was based upon the reaction of peroxides with ferrous iron and measurement of the resulting ferric iron as ferric thiocyanate. Shortly thereafter, Lips et al. (127) adapted the method to use with oils and fats. The values found by these methods were considerably higher than those by iodometric methods and since the method was more sensitive, it was thought by some that it was more exact, reacting with some peroxides with which the iodometric method failed to give a reaction. Other factors which were thought to be involved were reaction of iodine with ethylenic linkages, slow reaction in presence of moisture, and volatilization of iodine.

A study of the influence of atmospheric oxygen in the Chapman and McFarlane method was made by Lea (123) who found about a 75 per cent reduction in values by careful exclusion of air. He also found that the degree of further oxidation

occurring during the determination is roughly proportional to the quantity of peroxide already present.

After experiencing difficulty with the low solubility of fat in the 96 per cent acetone used as a solvent in the Chapman and McFarlane method, as well as trouble due to rapid oxidation of ferrous salts in the solvent, Hills and Thiel (98) modified the method to give what they considered sufficient sensitivity to follow the oxidation of the fat from the time it leaves the udder. The solvent used was made up of 70 volumes benzene to 30 volumes methanol. The FeSO_4 and NH_4SCN are added separately.

A comparison of the method of Hills and Thiel with that of Lips et al. caused Chapman and McKay (34) to conclude that the former was more sensitive. When the method of Lips et al. was compared with that of Hills and Thiel with oils, results by the former averaged 1.45 times the latter despite considerable oxygen interference in the Hills and Thiel method. Exclusion of air gave much lower values for both iodometric and ferric thiocyanate methods.

These same workers (34) made a comparison of the Lips et al. and Bolland et al. (20) thiocyanate methods with the Lea (124) "cold" method on H_2O_2 , three fatty acids, two esters, and two oils. While good agreement was obtained on H_2O_2 , the two thiocyanate methods gave higher results on the other materials than did the iodometric.

While all were affected, the interference by atmospheric oxygen was less in the Hills and Thiel method and the Bolland procedure than in the method of Lips et al. Of the methods investigated, the Hills and Thiel method was found to be the most satisfactory since the reagents are more stable and the benzene-methanol solvent allows larger sample size. With ferric thiocyanate methods, the results, while higher, are proportional to those by the iodometric; the former was more sensitive.

Kolthoff and Medalia (116) showed a scheme of reaction of hydroperoxide molecule with ferrous iron. Steps one and two show the stoichiometric reaction:



while step three shows a reaction competing with step two which could form a new radical



The new radical, R'^\bullet , can then either reduce ferric iron, as in step four where R'^\bullet is represented as CH_3CHOH (but could be some other radical if a different solvent were employed),



or react with oxygen (if present) to form a new peroxide radical



thereby initiating reactions according to this same scheme.



It is considered that the solvent can participate in this series, especially beginning at step three. Thus, some solvents are termed "promoting" solvents, others "suppressing" solvents; the reaction of the solvent may depend upon the particular peroxide. Acetone was considered a suppressor solvent. These authors consider the ferrous iron methods inherently less accurate than the iodometric, a statement which seems not too well documented. On the basis of their statement, they feel that iodometric methods are to be preferred for accurate work.

Since the reaction scheme shown above includes radicals at every step and is a chain reaction, this may account for Lea's observation (123) that the degree of oxygen interference is proportional to the amount of peroxide present.

Brief mention might be made of a recently developed peroxide method by Hartmann and Glavind (91) based on the reaction of organic peroxides with 3,5-dichloro-4,4'-dihydroxyphenylenediamine to form dichlorophenolindophenol when is then determined colorimetrically at 520 millimicrons. While the method has apparently not yet received much study, Hartman and White (90) found high value in the presence of air, but in the absence of oxygen the values were lower than iodometric values.

The results are fairly reproducible and can, therefore, be used for comparative purposes.

3. Carbonyl compounds

a. Application in fat oxidation. For many years, it has been recognized that carbonyl compounds were formed in the oxidation of fats and oils. In many cases it has been considered that the mechanism of their formation or even the exact compounds formed have not been known. There seems to be ample evidence of their formation; only some of the more recent literature will be cited here. Some further evidence was mentioned previously in discussing peroxide decomposition products.

Powers (172) lists five stages in the oxidation of drying oils, namely induction period, peroxide formation, peroxide decomposition, polymerization, and degradation. (The last two may not be particularly evident in non-drying oils.) He lists four types of peroxide decomposition, one of which is chain rupture with the formation of two aldehyde groups (which of course could undergo further oxidation to carboxyl groups). Spectrophotometric evidence obtained by Dugan et al. (45) indicate the formation (at different rates) of at least two carbonyl compounds. They did not identify the materials or even indicate whether they were aldehydes or ketones. In a study of the aldehydes produced in autoxidation of cottonseed

oil, Swift et al. (202) presented spectrophotometric and melting point data on both the semicarbazones and regenerated carbonyls to show that deca-2,4-dienal, oct-2-enal and hexanal were formed, the mechanism of which is shown on page 40. The same authors had earlier presented evidence (200) of the presence of analogs and homologs of undec-2-enal in the decomposition of methyl hydroperoxido oleate.

After steam distillates from rancid corn and avocado oils were collected into 2,4-dinitrophenylhydrazine, Brekke and Mackinney (23) were able to separate the mixture into saturated derivatives of saturated aldehydes, -enals, and dienals. Precise identification of each component was not made, but the corn oil fraction had at least five components while that from avocado oil had at least six.

In a study of methods for investigation of rancidity of bacon fat, White (217) found the peroxide oxygen, Kreis and aldehyde tests to be the most suitable of those tried (ten in all) for estimation of rancidity. The Kreis test is generally thought of as a test involving reaction with epihydrinaldehyde (the mechanism of formation of which has not been fully explained) although Patton et al. (168) seem inclined to believe it is malonic-dialdehyde. (Note: epihydrinaldehyde could probably change to malonic dialdehyde and vice versa merely by a molecular rearrangement.) In any case, it seems to be due to a carbonyl compound, although undoubtedly other

carbonyl compounds are also present.

Holm et al. (102) found oily-flavored butter to contain alpha, beta-unsaturated carbonyl compounds in quantities related to the flavor intensity.

Infrared spectrophotometric studies of autoxidizing milk fat caused Henick (94) to conclude that the CS₂ extract from the steam distillate contains at least one unsaturated conjugated ketone and another not conjugated. He felt that this examination was more sensitive than taste panels which were in turn more sensitive than chemical methods in the detection of deterioration.

Keeney and Doan (110, 111, 112) were able to isolate ketones which when added to fresh milk in very small quantities, gave it a flavor resembling oxidized flavor.

b. Methods of determination. Lea (122) prescribes a method for estimation of aldehydes wherein a benzene solution of the fat is extracted by shaking with aqueous sodium bisulfite solution. The excess bisulfite is reacted with iodine and after treatment with sodium bicarbonate, the bisulfite liberated from combination with the aldehyde is titrated with 0.002 N. iodine.

More recently, some colorimetric methods involving the use of 2,4-dinitrophenylhydrazine have been proposed. Pool and Klose (171) published a method for monocarbonyl compounds, wherein the hydrazones are formed on a chromatographic column

of alumina. The column removes excess reagent and the hydrazones of dicarbonyl compounds. The colorimetric measurement of the remaining hydrazones is made at 435 millimicrons. The sample size should be such as to contain 0.05-0.50 micromoles of aldehyde. While the authors did compare the color development of several saturated aldehydes, two unsaturated aldehydes and acetone, nothing is said in the discussion regarding the use of the method for ketones. Mention is made of the fact that the unsaturated ketones give a slightly different absorbency at a given concentration than do the saturated aldehydes. Acetone seems likewise to show a deviation.

Lappin and Clark (120) determined aldehydes and ketones in a concentration range of 10^{-4} to 10^{-6} mole per liter by measuring at 480 millimicrons the red color produced by the reaction with 2,4-dinitrophenyl-hydrazine in the presence of KOH. No separation is involved; the mixture is heated at 50° for 30 minutes or 100° for five minutes.

Schoniger and Lieb (188) suggest a titrimetric method involving hydrazone formation. The sample is reacted with 2,4-dinitrophenylhydrazine in the presence of HCl. The excess reagent is treated with a known amount of $TiCl_3$ and the excess of this latter reagent titrated with ferric alum to a thiocyanate end point.

A less sensitive method involving differential pH measurements was suggested by Roe and Michell (180). The

results are said to be reproducible to plus or minus two per cent.

4. Iodine value

a. Application to study of fat oxidation. After oleic acid had absorbed one atom of oxygen per molecule, the reaction slowed down considerably and Meyer (136) found by iodine number determination that the double bond remained almost unchanged.

Farmer and co-workers (54, 57) feel that in the primary product of reaction with oxygen, the double bond remains unchanged and, therefore, the iodine value should be unchanged. They were able (59) to isolate the hydroperoxide of cyclohexene and found that it would still absorb one molecule of bromine as before.

However, these workers do note (57, 59) a decrease in iodine number with increasing oxidation. This point is well substantiated by other workers.

Hilditch and Sleightholme (97) and Banks and Hilditch (11) noted quite large decreases in iodine number upon oxidation, in one instance the value on an olive oil dropping from 83.6 to 9.2 (although upon heating under vacuum it rose to 17.7).

Hess and O'Hare (95), Farmer and Sundralingam (60), and Skellon (192) noted drops in iodine value, the latter worker finding the value to drop rapidly after the end of the

induction period.

Atherton and Hilditch (6) and Gunstone and Hilditch (84) noted drops in iodine value upon oxidation at temperatures ranging from 20° to 120° with the drop much more pronounced at the higher temperature. The findings of Feuill and Skellon (65) are in agreement with this and on this basis, they feel that a different oxidation mechanism is operative at higher temperatures.

After allowing methyl hydroperoxido-oleate to react with oleic acid and then separating the ester from the acid, Swift and Dollear (199) noted that the iodine value of the ester had dropped from 69.4 to 66.6. This is explainable on the basis of intramolecular oxidation of the double bond with reduction of the hydroperoxide group.

The drop in iodine value was found by Franke and co-workers (150, 151) to parallel the oxygen absorption by unsaturated fatty acids to an uptake of about 25 per cent, after which it dropped more slowly.

For approximately linear portions of the curves, Gunstone and Hilditch (84) calculated the rate of increase of peroxides in units per hour and the rate of decrease of iodine value in units per hour for methyl oleate at various temperatures from 20° to 130°. The results are shown in Table 3.

Assuming a cyclic peroxide structure, Paschke and Wheeler (167) calculated a theoretical peroxide value based on

Table 3

Rate of Change of Peroxide and Iodine Values
With Change in Temperature^a

| | Units change per hour at | | | | | |
|-----------------------------|--------------------------|------|-----|------|------|------|
| | 20° | 50° | 80° | 100° | 120° | 130° |
| Peroxide value, increase | 1.2 | 2.9 | 28 | 110. | 330 | 450 |
| Iodine value, decrease | 0.009 | 0.03 | 1.7 | 5.5 | 9.0 | 10.0 |

^aTaken from a table by Gunstone and Hilditch (84, p. 837).

iodine number decrease. In the early stages this theoretical value agreed well with the observed peroxide value, the lower the temperature, the higher the value before deviation was apparent.

In the early stage of autoxidation of methyl linoleate the fall in iodine value observed by Gunstone and Hilditch (84) is nearer to that caused by formation of a hydroperoxide at an alpha-methylene group than that due to attachment of oxygen at a double bond. However, the observed drop in iodine value soon approaches and passes that calculated on the latter assumption (and more rapidly than with oxidized methyl oleate). These workers feel that this is to be expected since in addition to failure of peroxide value determination to indicate total peroxides produced, there is an increasingly

significant proportion of conjugated esters formed and these do not register true iodine values under Wijs conditions.

The discrepancy between Wijs and Hübl iodine values is considered by Morrell and Phillips (144) to be due to enolization of ketol groups. Carrick et al. (33) believe that the difference between the Woburn and Wijs values gives an approximate measure of conjugated diene value of the oils. However, when spectral absorption of oxidized methyl linoleate showed 1.48 molecules of double bond in conjugated form, Lundberg et al. (132) obtained a Woburn iodine value indicating 1.68 molecules double bond, while the Wijs value showed 1.69 moles of double bond. These authors comment that this seems unusual in view of the fact that the Wijs method is known to give low values with conjugated substances.

After a chromatographic separation of autoxidized methyl linoleate, Dugan et al. (44) considered that the most highly oxygenated substances (though not necessarily those of highest peroxide value) are more tightly held and thus are nearer the top of the column, while the material nearer the bottom seems to have progressively higher iodine values.

In twelve trials during winter months of two successive years, Brown et al. (25) could find no measurable change in the iodine number of milk fat as a result of the development of moderate or fairly pronounced oxidized flavor. These workers believed that the oxidized flavor was due to

oxidation of phospholipids and they showed that even if all the lecithin normally found in milk fat were oleo-stearo lecithin, its complete oxidation could not measurably change the iodine number.

The results obtained by Brown et al. (26) indicate that there is a direct relationship between the iodine number of the fat in the ration and the intensity of the metal induced oxidized flavor. The feeding of one pound per day of coconut oil decreased slightly the iodine number of the resulting butterfat and reduced slightly the intensity of the oxidized flavor developed by copper. The feeding of soybean oil increased greatly the iodine number of the butterfat and increased the susceptibility of the milk to oxidized flavor. The results from control cows on low fat rations indicate little correlation between iodine number of the butterfat and the intensity of metal-induced oxidized flavor.

In contrast, Corbett and Tracy (38) found that the feeding of corn or coconut oil markedly altered the iodine number of the fat, but produced only a slight change in susceptibility of milk to oxidized flavor. The susceptibility of milk to development of oxidized flavor was not correlated to the iodine number in the range of 24 to 40. Milk with an iodine number greater than 40 developed oxidized flavor to a slightly greater degree than milk having a lower iodine number, but the differences did not appear to be significant.

b. Methods. Breazeale, working in this laboratory made a detailed critical study (22) of halogen addition reactions. He compared the Hanus, Wijs, Rosenmund-Kuhnhenh (185), Kaufmann, ^Hübl and modified ^Hübl methods; in the last the reagent was employed in methyl alcohol. Of the methods studied, he deemed the Rosenmund-Kuhnhenh the most reliable. He considered that the bromine vapor method should give values very closely representing only the degree of unsaturation of the fat and on this basis, concluded that the Rosenmund-Kuhnhenh method gave very nearly the correct iodine number. He found with the other methods a greater tendency for the values to increase as reaction time increases. On the basis of his work, the Rosenmund-Kuhnhenh method was selected for use in the present study.

However, a few citations from more recent literature will be made here. A comparison of Wijs, Hanus, Rosenmund-Kuhnhenh and Kaufmann methods was made by Paschke and Wheeler (167) on distilled methyl esters of soybean fatty acids. Their results are presented in Table 4. It is interesting to note that while the Rosenmund-Kuhnhenh method gives the lowest value in all cases and also gives a greater drop in iodine value on oxidation than the Wijs or the Kaufmann, the drop shown by the Hanus method on oxidation of the esters is appreciably greater than even that of the Rosenmund-Kuhnhenh method.

Table 4

Iodine Number, Comparison of Methods^{a,b}

| | Method | | | | | |
|-----------------------------|--------|----------|-------|----------|---------------------|----------|
| | Wijs | | Hanus | | Rosenmund-Kuhnemann | |
| | Fresh | Oxidized | Fresh | Oxidized | Fresh | Oxidized |
| Iodine value | 131.2 | 117.6 | 129.8 | 111.8 | 106.4 | 116.7 |
| Reaction time ^c | 30 | 30 | 30 | 30 | 5 | 120 |
| Excess reagent ^d | 200 | 200 | 200 | 200 | 150 | 200 |
| Iodine value | 130.3 | 116.8 | 128.4 | 109.1 | 104.2 | 116.4 |
| Reaction time | 30 | 30 | 30 | 30 | 5 | 120 |
| Excess reagent | 100 | 100 | 100 | 100 | 33 | 100 |
| Iodine value | 130.6 | 117.2 | 131.3 | 113.9 | 108.6 | 117.8 |
| Reaction time | 60 | 60 | 60 | 60 | 10 | 240 |
| Excess reagent | 200 | 200 | 200 | 200 | 150 | 200 |
| Iodine value | 130.2 | 116.7 | 130.0 | 111.5 | 105.5 | 117.3 |
| Reaction time | 60 | 60 | 60 | 60 | 10 | 240 |
| Excess reagent | 100 | 100 | 100 | 100 | 33 | 100 |

^aReproduced from Paschke and Wheeler (167, p. 53).^bComparisons on fresh and oxidized distilled methyl esters of soybean fatty acids.^cReaction time expressed in minutes.^dExcess reagent expressed as per cent of reagent reacting.

Various workers have attempted to reduce reaction time in iodine value determinations by the use of accelerators or catalysts. Rosenbusch and Parker (184) applied the use of mercuric acetate to the Hanus method on castor oil. The results were higher than with the standard method and the deviation increased with increased mercuric acetate. By reducing the amount of catalyst, they were able to obtain values giving fairly good agreement with the standard method.

In a discussion of various methods, Benham and Klee (12) found the Rosenmund-Kuhnhehn reagent satisfactory by reason of (a) ease of preparation, (b) stability, and (c) versatility (noting that both Wijs and Hanus methods are unsatisfactory for conjugated systems). These workers recommend the use of 10 ml. of 2.5 per cent HgCl_2 in acetic acid as a catalyst. They consider that constant values were obtained with 100 per cent excess reagent, although their figures would seem to better support the use of 150 per cent excess. They likewise claim no increase in absorption after one minute with catalyst. However, their figures on ten of eleven oils show increases which would be equivalent to 0.3 - 1.3 iodine units in 60 minutes. In a comparison of their modified Rosenmund-Kuhnhehn method with the Hanus, Wijs and standard Rosenmund-Kuhnhehn methods, they obtained the results shown in Table 5 (the two oils used contained large amounts of conjugated double bonds). It may be noted that the lowest results are obtained by the

Table 5
Comparison of Iodine Values^a

| Oil or acid | Iodine value by | | | |
|----------------|-----------------------|-------|-----------------------|-------|
| | Mod. R-K ^b | Hanus | Std. R-K ^c | Wijs |
| Linoleic acid | 178.8 | 179.7 | 173.2 | 180.7 |
| Linolenic acid | 268.5 | 267.9 | 261.2 | 272.5 |
| Oiticica oil | 195.4 | 195.2 | 134.4 | 144.8 |
| Tung oil | 229.4 | 216.3 | 156.7 | 162.4 |

^aTaken from a table by Benham and Klee (12, p. 129).

^bModified Rosenmund-Kuhnhehn method.

^cStandard Rosenmund-Kuhnhehn method.

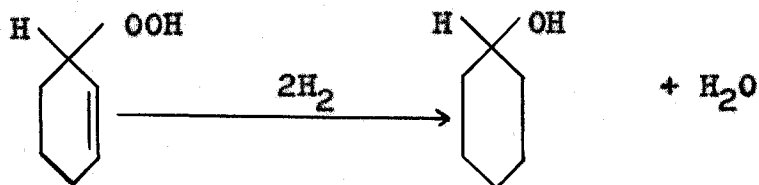
standard Rosenmund-Kuhnhehn method, while with the conjugated oils the results by the Wijs method are almost as low.

A sample of tung oil stored several years without precautions against deterioration gave a value (115) by the modified method which was about seven or eight units below that found on several fresh samples, while that by the standard Rosenmund-Kuhnhehn method was only one or two units below that of fresh samples. Their modified method used on beta-elostearic acid with 350 per cent excess and one, two and 48 hours reaction time gave values of 272.1, 273.3 and 272.8 respectively, compared with a calculated value of 273.7. With conjugated soybean acids (one hour and 150-640 per cent excess reagent) a value of 138.5 was obtained, being the same as that

before isomerization. The Wijs method (one hour and 390 per cent excess) gave 117.5 on the conjugated acids.

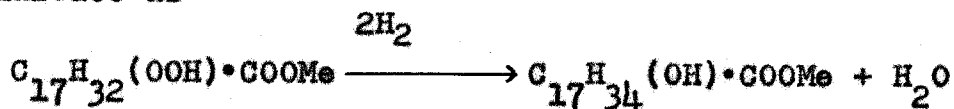
5. Hydrogenation value

a. Application to a study of fat oxidation. After isolating a peroxide of cyclohexene, Farmer and Sundralingam (59) hydrogenated it (method not given except to say that Adams catalyst, $\text{PtO}_2 \cdot \text{H}_2\text{O}$, was used). The material was found to absorb two molecules of hydrogen, giving cyclohexanol and not cyclohexandiol. Thus, the reaction must have been



Ultra violet light caused a drop of about half in the peroxide oxygen content and in the amount of hydrogen absorbed. This can be explained on the basis of half the peroxide groups reacting intramolecularly or intermolecularly with half the double bonds.

Farmer and Sutton (62) concentrated a hydroperoxide of methyl oleate which (although admittedly not free of impurities) absorbed hydrogen in a manner which these researchers indicate as



The actual amount of hydrogen taken up varied with different

preparations from 1.97 to 1.86 molecules hydrogen per molecule of ester.

Allen et al. (2) oxidized fatty acid esters and at various stages, removed samples for hydrogenation in a modification of the apparatus of Johns and Sieferle (107). Up to the point at which 0.2 molecules oxygen per molecule of methyl linoleate had been absorbed, they considered all the oxygen to be in the form of peroxide and by correcting the hydrogen uptake for peroxide oxygen (subtracting molecules of peroxide from molecules hydrogen) they found the hydrogen uptake constant at (about) two molecules hydrogen per molecule of ester. Beyond this there was a considerable increase in nonperoxide oxygen and a decrease in hydrogen uptake. Whether this latter is due to interaction of hydroperoxide with double bond to form hydroxy and epoxy groups or to chain scission or both, is not indicated. It is of particular interest to note that the uncorrected hydrogenation value increased in the early stages of oxidation, whereas, although these researchers did not show it, the iodine value would not be expected to change until such time as the corrected hydrogen value dropped.

The question might logically be raised, in view of the fact that carbonyl compounds have been indicated as being present in oxidizing fat, as to whether aldehydes or ketones would be reduced under the conditions used. Csuros and Sello (41) claim that while aldehydes linked to an aromatic ring

could be easily hydrogenated, aliphatic aldehydes and ketones could not be hydrogenated at the carbonyl group with palladium on bone black or with colloidal platinum or palladium.

In agreement with this, Swift et al. (202) isolated carbonyl fractions which they identified as deca-2,4-dienal, oct-2-enal and hexanal. After hydrogenation (not quantitative) they were still able to identify the materials as the corresponding saturated aldehydes.

However, Carothers and Adams (32) had found that the carbonyl group of benzaldehyde and heptaldehyde could be reduced with Adams catalyst provided a promoter in the form of ferrous (or ferric) chloride is added. Without this promoter, the catalyst quickly becomes inactive when shaken with an aldehyde. Their interpretation is that the aldehyde, being so readily oxidizable deprives the catalyst of its oxygen, which oxygen is necessary for the catalytic activity. The iron salt specifically inhibits this reaction.

b. Methods. According to Vandenheuvel (212a), with few exceptions all laboratory hydrogenation apparatus has been derived from the reaction vessel-buret-leveling bulb system, and while many modifications have been suggested it was felt that a precision of better than one per cent could not be expected. There are, however, some exceptions.

Hyde and Scherp (106a) describe a hydrogenation apparatus wherein the amount of hydrogen absorbed is measured by

pressure change (as in Barcroft-Warburg apparatus). However, instead of the equipment being open to the atmosphere on one side of the manometer, it connects with a control vessel having the same volume as the reaction flask. Each manometer arm was graduated with a metric scale. It should be noted that this apparatus is neither constant volume nor constant pressure. While an accuracy of ± 2 per cent is claimed, it was noted that one of the six recorded results gave 104 per cent of the theoretical value.

In 1940, Prater and Haagen-Smit (173) developed an apparatus consisting of three sections, (a) hydrogen purification, (b) reaction and measuring, and (c) vacuum system. The reaction and measuring section consisted of two symmetrical parts connected by an equalizer tube and a manometer. The entire section is mounted on a board and rocked by an eccentric at 75-150 strokes per minute through a 15° arc. Both the reaction flask and the measuring flask are charged with solvent and catalyst. After the catalyst has been reduced, the pressure is equalized on both sides.

Another hydrogenating system mounted on a swinging board was designed by Johns and Seiferle (107). However, this system included a gas buret and was thus a constant volume-changing pressure design. These authors consider that the amount of hydrogen absorbed by the catalyst can be calculated on a theoretical basis so that when an accuracy of ± 6 per cent

is satisfactory, sample and catalyst can be reduced simultaneously, saving time and making use of a more vigorous catalyst. But even where the hydrogen absorption by the catalyst is measured, the claim is only for an accuracy of ± 2 per cent.

Noller and Barusch (164) also prefer to reduce catalyst and sample simultaneously. However, they use a different method of arriving at a correction. They equilibrate with sample, but no catalyst in the flask and then add catalyst, using a blank having solvent and catalyst to obtain the correction. Their apparatus also uses a gas buret, but rather than shaking the entire apparatus, they use a magnetic stirrer. No figures were given by them as to the accuracy of the method.

An apparatus wherein the sample need not be in the system during the equilibration, was described by Orchin and Weber (166). They introduced the sample through a neoprene stopper by means of a hypodermic syringe. A gas buret and magnetic stirrer are included, but no provision is made for temperature control.

The apparatus described by Ogg and Cooper (165) differed from the ones just described mainly in having the leveling bulb mounted on a rack and pinion. However, their procedure differed in some respects. The hydrogen was purified by passage through a combustion tube (750°) containing platinum

catalyst and then through indicating drierite after which it was saturated with the solvent (acetic acid). No mention was made of applying correction for barometric changes nor was provision made for precise temperature control. Their values on fatty acids and esters were generally within one per cent of the theoretical values.

Kaufmann and Baltes (109) also used the gas buret principle. Their apparatus, housed in a constant temperature box, provided for the shaking of the entire reaction flask-gas-buret-leveling bulb combination. Their sample size was small (20-40 mg.) and the design of the reaction flasks such that a small amount of the sample could easily adhere to the flask where it would not come into contact with the catalyst. The values they report on linoleic and elaidic acids while covering a range of three parts per thousand, are 1.5-2.0 per cent below the calculated values. On linoleic acid, the range of values (about 9 parts per thousand) includes the calculated value. Acetic acid was used as a solvent, but difficulty was mentioned, the saturated acids not being particularly soluble in this.

A slightly different approach to the basic principle of a gas buret was used by Clauson-Kaas and Limborg (36). They have two similar vessels connected by an equalizer tube and a differential manometer. After the catalyst has been hydrogenated, the pressure in the two vessels is equalized and the

stopcock between the two closed. After the sample, which was in a cup dropped from a hook by a magnet, has been hydrogenated, mercury is added from an attached buret into the hydrogenation vessel until the pressure in the two vessels is equal. The amount of mercury added indicates the amount of hydrogen absorbed. The figures presented as to the precision of the method were on sorbic acid and differed from the calculated value by not more than 0.2 per cent on five samples.

Vandenheuvel (212a) described an apparatus in which he claimed that pressure is automatically maintained within 0.03 mm Hg of the preset pressure. Actually, his description would seem to indicate that this is on the assumption that barometric pressure remains constant, which it certainly does not. His device is a variation of the reaction vessel-buret-leveling bulb system wherein the leveling is done by electromagnetically lifting a needle valve, the magnet being controlled by a contact in an open end manometer. Agitation is provided by means of several disks mounted on a central tube. A pulsating current pulls the agitator down against a coil spring at about 300-500 pulsations per minute. Results of 30 runs on fumaric acid in 95 per cent ethanol range from 1.733 grams hydrogen per 100 grams sample to 1.742, with a value of 1.7374 calculated by the author. One of the main advantages claimed by this worker is that the apparatus may be used for kinetic studies since readings may be taken as often as every

15 seconds.

The Warburg apparatus was used by Mead and Howton (135) in hydrogenation of less than milligram amounts of unsaturated fatty acid derivatives. They used the standard 15 ml. single sidearm Warburg flasks. Ethanol (3 ml.) was used as a solvent while 5 mg. of palladium black on charcoal served as a catalyst. Corrections were made against a manometer reading on a flask containing no unsaturated material. The figures given show deviations from calculated values ranging from 2.2 per cent to 7.7 per cent. However, it must be noted that these workers were using samples of less than four micromoles.

The use of Warburg apparatus for quantitative hydrogenations as well as for other manometric measurements is mentioned in various discussions of this apparatus (73, 169). Its use in the study of oxidation of fats is described in numerous articles (9, 103, 108, 129 and 159, as well as many other articles mentioned in various abstracting journals). For example, Banks (9) and Lundberg and Chipault (129) studied the effects of antioxidants by this means, while Johnston and Frey (108) used it to measure an induction period.

It might be noted that in a large number of the so called quantitative experiments recorded in the literature, the researchers have apparently been interested in working with rather pure compounds in an endeavor to find the number of double bonds or else have for other reasons apparently been

satisfied with errors of one or two per cent.

Many of the articles on hydrogenation do not specify what catalyst has been used. Orchin and Weber (166) used a platinum oxide catalyst, but do not specify the method of preparation. Clauson-Kaas and Limborg specified that they used Adams (213) catalyst and apparently found it satisfactory. In both these cases, magnetic stirrers were used. Mead and Howton (135) discontinued the use of Adams PtO_2 catalyst because the small amounts used were not adequately dispersed by the shaking typical of the Warburg apparatus.

The Adams catalyst is prepared (213) by fusing chloroplatinic acid with sodium nitrate and keeping the melt hot until evolution of the oxides of nitrogen has practically ceased (usually five to fifteen minutes). This is followed by washing the precipitate (with water) to free it of nitrates. If the precipitate is not thoroughly dried, it generally is not a particularly active catalyst. The best temperature for holding the fusion mixture was considered by Voorhees and Adams (213) to be about 450° . In a later paper, Adams and Shriner (1) favored a temperature of 500° . Frampton et al. (68) preferred a temperature of 520° , but pointed out that catalysts prepared at 500° and 540° were almost as active. Temperatures above 540° gave decreased activity. Frampton et al. feel that the procedure of Voorhees and Adams does not always produce an active catalyst, while their procedure,

which includes adding a dry mixture of 1 g. chloroplatinic acid and 9 g. NaNO_3 to a fusion mixture of 100 g. NaNO_3 , as well as a closer control of temperature, allows a more uniform preparation.

When Mead and Howton (135) discontinued the use of Adams catalyst, they began using 10 per cent palladium black on charcoal, to which they indicated no objections. Ogg and Cooper (165) had used palladium (concentration not given) on activated carbon.

While Voorhees (213) and others had objected to the use of platinum black or palladium black, Vandenheuvel (212a) used the former with apparently good results. However, for some kinetic studies on the hydrogenation of methyl oleate, Vandenheuvel used Raney nickel. It is possible that this latter catalyst may be enough less active to allow the course of the reaction to be more easily followed.

III. EXPERIMENTAL

A. Development of a Hydrogenation Method

1. General considerations

a. Reasons for using hydrogenation. There are two main reasons for wanting to quantitatively hydrogenate milk fat. The results obtained by the various iodine number methods do not show agreement and, even with a given method, the results obtained will vary with the conditions of the determination. These variations may be due either to incomplete reaction, giving low results on the one hand, or to side reactions and substitutions, giving high results on the other hand. Hydrogen, being a smaller molecule than the halogens, and under the proper catalytic influence being highly reactive toward ethylenic linkages, should bring about complete saturation of double bonds. No interference should be expected from conjugated linkages (possibly arising from oxidation) with hydrogen, while such is known to occur in some halogen addition methods. Substitution would pose no problem. There is one "side reaction" which would be expected in a peroxidized fat but that is the basis for one of the reasons for using hydrogenation. If the peroxides are in the form of hydroperoxides, these should react with hydrogen to form a hydroxyl

group and a molecule of water, each molecule of hydroperoxide requiring one molecule of hydrogen. Thus, it is hoped that hydrogenation will give (a) on a fresh fat, a true measure of unsaturation and (b) on an oxidized fat, a measure of hydroperoxides plus the then existing unsaturation.

b. Reasons for desiring a new method. As was mentioned in the Review of Literature, the majority of the methods in use for hydrogenation give a reproducibility of not better than plus or minus one per cent. None of those described except the one using the Warburg apparatus (135) and the Kaufmann-Baltes (109) apparatus are adaptable to simultaneous determinations. In many, the methods of controlling temperature or correcting for barometric changes were either absent or inadequate. The Kaufmann-Baltes apparatus, using an air bath, was not feasible for use at a temperature necessary to keep the fat liquid, especially after the fat has been saturated. At lower temperatures, the reaction does not proceed because the fat solidifies around the catalyst. Thus none of the methods described adequately meet the requirements of this study. It was therefore decided to attempt to devise a method which would more nearly meet the requirements.

c. Characteristics desired in a hydrogenation. While it was recognized that there would be difficulties in the achieving of all of them, several desirable characteristics may be listed for the procedure to be devised. Some of these are:

- (1) Precision and reproducibility of reading.
- (2) Ease of reading.
- (3) Good temperature control in proper range.
- (4) Adequate agitation.
- (5) Ease of calculation of results.
- (6) Rapidity of determination.
- (7) Absence of side reactions.
- (8) Simultaneous multiple (battery) determinations.
- (9) Accommodation of sample of adequate size.
- (10) Simplicity in care, cleaning and operation of equipment.

d. Presentation of material. One of the two principal parts of this thesis is concerned with the development of a quantitative hydrogenation method. This development was spread out over several years but during this time numerous hydrogenations were made. These determinations, while not precise, probably in most cases did not vary more than 0.5 to 0.75 iodine unit and therefore have some value. However, since there were factors (undetermined during most of the development phase) which prevented the attaining of equilibrium pressures either before or after actual hydrogenation, it was not possible in many cases to make an accurate study of the effect of varying certain factors. As a result, while changes were made from time to time, these were not made in an order suitable for outlining in definite chronological steps. In

other words, in many cases, it was necessarily a trial and error procedure. For this reason, the presentation of the material on this developmental phase is somewhat in narrative style, with the inclusion of such data as seem pertinent and reasonably valid. Furthermore, it is presented in what seems a logical sequence. It is realized that taking the material out of chronological order in a developmental procedure has its disadvantages. While most of the changes made were considered improvements, it has not always been possible to demonstrate beyond doubt that they were necessary. A further disadvantage is that it is not possible to say in each case that only this or that factor has been varied.

2. Hydrogenation apparatus

The Warburg apparatus seemed to be the most promising equipment design in regard to meeting the above listed characteristics, although it was recognized from the beginning that certain modifications would be necessary.

a. Reaction flasks.

(1) Design. Since the conventional Warburg manometer has a 300 mm. scale, it was desired to use a sample of such size that the change in hydrogen pressure would be roughly 200 mm., leaving a total of 100 mm. leeway in the original reading (since initial pressure cannot be precisely controlled) and in the final reading (since the change will depend upon

hydrogenation value as well as sample size). On a sample with an iodine number of about 34-40, a pressure change of 200 mm. should be obtained (if mercury is used in the manometers) when the sample size is about 7 to 6 mg. per milliliter of gas space. Thus with the Warburg flasks usually used in physiological respiration studies, having a volume of 16-17 ml. exclusive of contents, the sample size would be limited to roughly 100 mg. or less. A larger sample would decrease the relative error in results due to any error in sampling or weighing. Also a given absolute error in volume calibration would result in a smaller relative error. A larger flask having a gas volume of 100 ml. would allow use of at least 600 mg. sample.

As originally designed and constructed, the flasks were about 45 mm. diameter (exclusive of side arm) and 100 mm. overall height, with a total volume of 110-135 mm. The flask itself was in two sections, a lid and a body, with a 45/12 standard taper joint. At the top of the lid was a 14/20 standard taper joint for mounting onto a standard Warburg manometer. A side arm about 16 mm. square and 30 mm. long was mounted about 40 mm. above the bottom, sloping downward at about 12°. These flasks were satisfactory except for the fact that the 45/12 joint was difficult to separate after the completion of a determination. As a result of the breakage of most of these in the process of opening them, the next group was

designed with a butt joint similar to that of a desiccator. As actually constructed, the sidearms on the second group were slightly larger but shorter and the flasks were slightly taller with a smaller inside diameter. Except for these differences, they were similar to the first group. It had been anticipated that the lid would be held to the body of the flask by means of three small tension springs. Since the springs did not prove satisfactory, it became necessary to use three small C-clamps on each flask. Except for necessitating considerable additional time in assembling the equipment this was satisfactory. Shortly before the completion of the present experiments, a third group of flasks were received. These were similar to the first group except for being taller and having longer ground zone in the joint, the joint being a 45/25 standard taper. These joints were easily held together with springs and easily parted on completion of hydrogenation.

(2) Calibration. The calibration of the volumes of the flasks was done by weighing the amount of mercury or of water. The flasks were cleaned, the lids were greased and secured in the usual manner. The flask-lid combination was weighed (estimated to nearest 0.05 g.), and was filled with liquid, the amount being adjusted to that just sufficient to bring the level to a given mark on the manometer against which the flasks were calibrated. (Note: By starting with this same manometer when calibrating manometers and proceeding in

the manner described, the flasks and manometers may be used in any combination.) As soon as the amount of liquid is properly adjusted, the temperature to the nearest 0.1° is recorded, following which the flask and contents are weighed. Then the flask is emptied, the joint parted and the grease removed. The entire procedure is repeated twice more, giving a total of three weighings, with the joint being greased separately for each weighing. Since it is essentially impossible to get exactly the same thickness of grease film each time, one then gets the average of three trials.

The weights of fluid were reduced to in vacuo weight to give a volume at the calibrating temperature (according to table "True capacity of glass vessels from the weight of the contained water or mercury when weighed in air with brass weights" (99)) and this volume was converted to 60° using a cubical coefficient of expansion of 1×10^{-5} for pyrex glass.

When the flask is used, the gas volume is less than the calibrated volume by the displacement of sample, solvent, catalyst and glass contents. The beads used in the flasks are sufficiently uniform in size that the weight of any ten of them is not likely to differ greatly from any other ten beads so the ten beads were assumed to weigh 2.15 g. The weight of the sample vial and catalyst vial can be obtained when weighing sample and catalyst. From the total weight of glass and its specific gravity, 2.24, (119) the displacement of the

glass may be calculated. Since the solvent is measured, its displacement at the reaction temperature can be calculated from the temperature at which it is pipetted and the equation for its thermal change in density. In the case of acetic acid this equation was found (160) to be

$$d_t = [d_s + 10^{-3} \alpha (t - t_s) + 10^{-6} \beta (t - t_s)^2 + 10^{-9} \gamma (t - t_s)^3] \pm 10^{-4} \Delta$$

In this equation d_t and d_s are densities in g. per ml. at the desired temperature (t) and at 0° (t_s) respectively. The values of d_s , α , β , and γ are 1.0724, -1.1229, +0.0058 and -2.0 respectively. According to the manufacturer of the propionic acid its coefficient of expansion was 0.001102 ml./ml./ $^\circ\text{C}$. By similar calculations, the displacement of the fat can be calculated from its weight and a density of 0.8896 g. per ml. at 60° (106b).

(3) Joint sealer. In order to obtain a gas-tight system and still allow the joints to be parted, some lubricant-sealer is needed on the ground glass joints. To be satisfactory, a sealer must be resistant to oxidation or reduction, stable toward heat at reaction temperatures, insoluble in the solvent used for hydrogenation samples, insoluble in mineral oil, provide a seal which will remain gas tight under the conditions of evacuation and hydrogenation, and allow the joint to be easily parted. Zaletel and Bird (218) found Celloseal

to have an iodine value of 74.18 while Celvacene (heavy) had a value of 67.01. The hydrogenation iodine values appeared somewhat higher. These workers then chose a mixture of Dow-Corning high vacuum silicone grease and Floridin. The high vacuum grease had an iodine value of 1.00 and a hydrogenation iodine value of approximately 2.06 when mixed with the Floridin. The Floridin was used to give the grease more body so it would not channel under the conditions of use. In the early part of the work developing the method, much difficulty was experienced in obtaining constant pressure in the flasks. At one time it was thought to be due to the sealer being used but later other factors were found. After these were corrected, generally good results were found but on one occasion when poor results were obtained, it was also noted that the silicone grease being used had a definite foreign odor, suggestive of maple syrup. The silicone was from an unopened container. When silicone from another container not having the foreign odor was used good results were obtained, pointing up the fact that not all lots of silicone grease are satisfactory. The same odor was noted in other containers bearing the same lot number as that of the grease giving the poor results.

The Floridin used to give the grease more body was sieved through a 150 mesh screen. Since Celite, a 300 mesh diatomaceous earth was being used in another connection, and since, being finer, it should permit a better seal while still giving

body to the sealer, it was substituted for the Floridin. In the later part of the work the sealer consisted of a mixture of six parts of silicone high vacuum grease to one part (by weight) of Celite.

However, this sealer could not be used on the syringes attached to the manometers since it was not possible to get a thin enough film to allow the plunger to slip into the barrel of the syringe without splitting the barrel. Consequently, on the syringe which formed the mercury reservoir, and on the stopcock just above it, Apiezon N grease was used.

b. Manometers

(1) Design. In respiration studies and other work with Warburg apparatus, the flasks are often merely flushed with the gas to be used. That was not considered satisfactory in this study for two reasons. In the first place there was no vent opening in the flasks. In the second place it was felt that flushing would not do a satisfactory job of removing all oxygen, especially that dissolved in the fat or solvent. If the air was to be removed by vacuum, it was necessary to remove it through the manometer. Furthermore, unless the pressure on the two sides of the manometer is equal, the manometer fluid will be pulled out of the manometer. Consequently manometers, with standard taper joints at the top of both arms, were designed so a manifold could be attached. When there were indications later that air was being pulled

through the wall of the rubber tubing mercury reservoir, a stopcock was placed on each manometer between the reservoir and the arms of the manometer; the stopcock was closed during the evacuations.

The mercury column was difficult to read against a light background; it was found that covering the back half of each manometer with black masking tape made the top of the column easier to see but with black filled etching on the scales, reading was not improved. However, since the filling was removed from the etched scale markings during cleaning, it was found that by painting the scales with white paint and removing the excess with a cleansing tissue, an easily readable scale was obtained.

While many attempts at hydrogenation were made over a period of months and results were obtained which had some value, the results could not be considered exact because it was not possible to obtain what was considered good equilibration either before or after the actual hydrogenation. In Figure 1 is shown the results of variations in several flasks, each set up in the manner of the usual thermobarometer. In Figures 2, 3 and 4, these same results are shown with various corrections.

Some difficulties had been experienced with gas penetration of rubber tubing so tygon tubing had been used to replace it as the mercury reservoirs and in the evacuation and filling

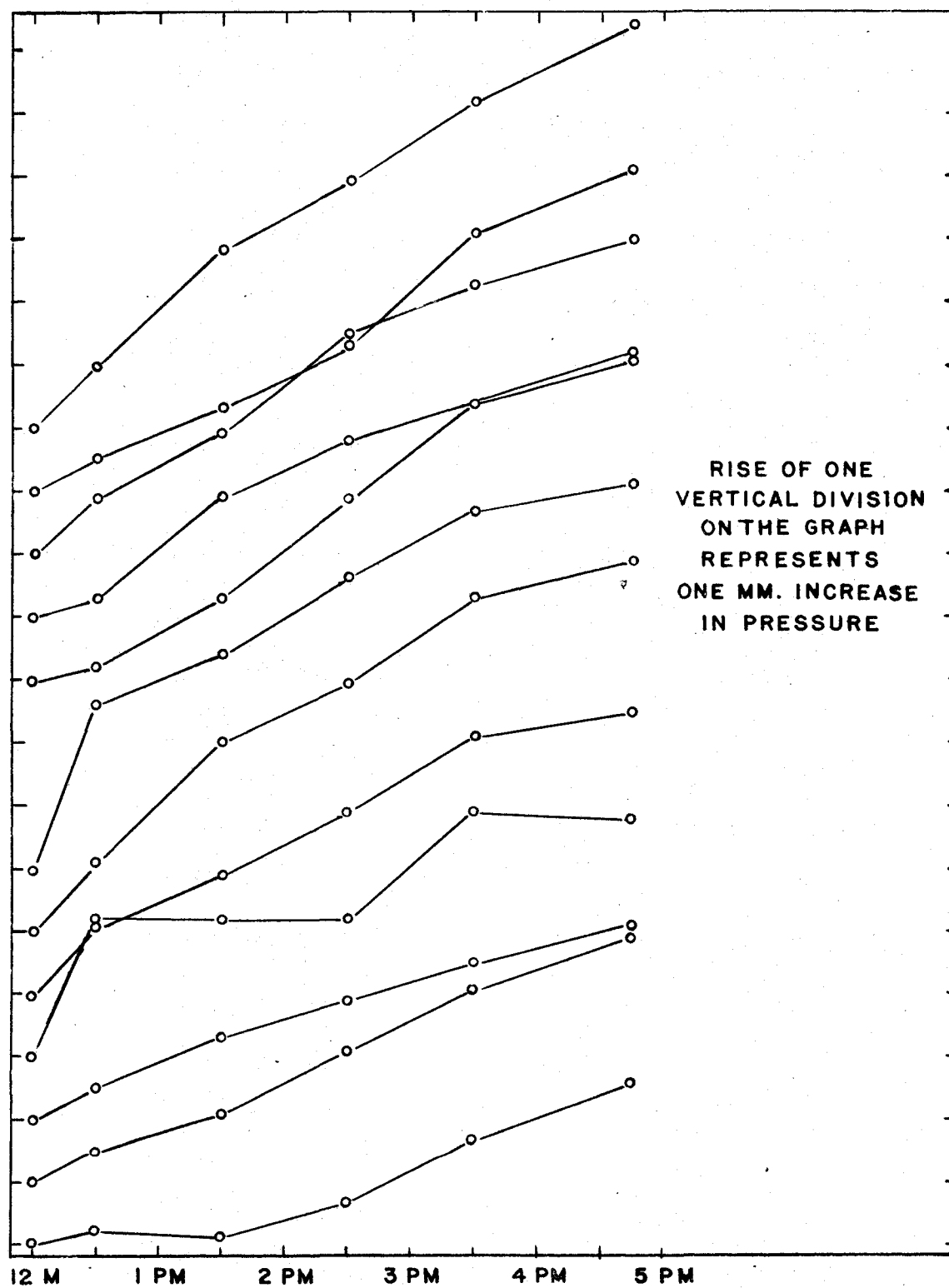


Figure 1. Pressure changes (uncorrected) in flasks having no sample solvent or catalyst.

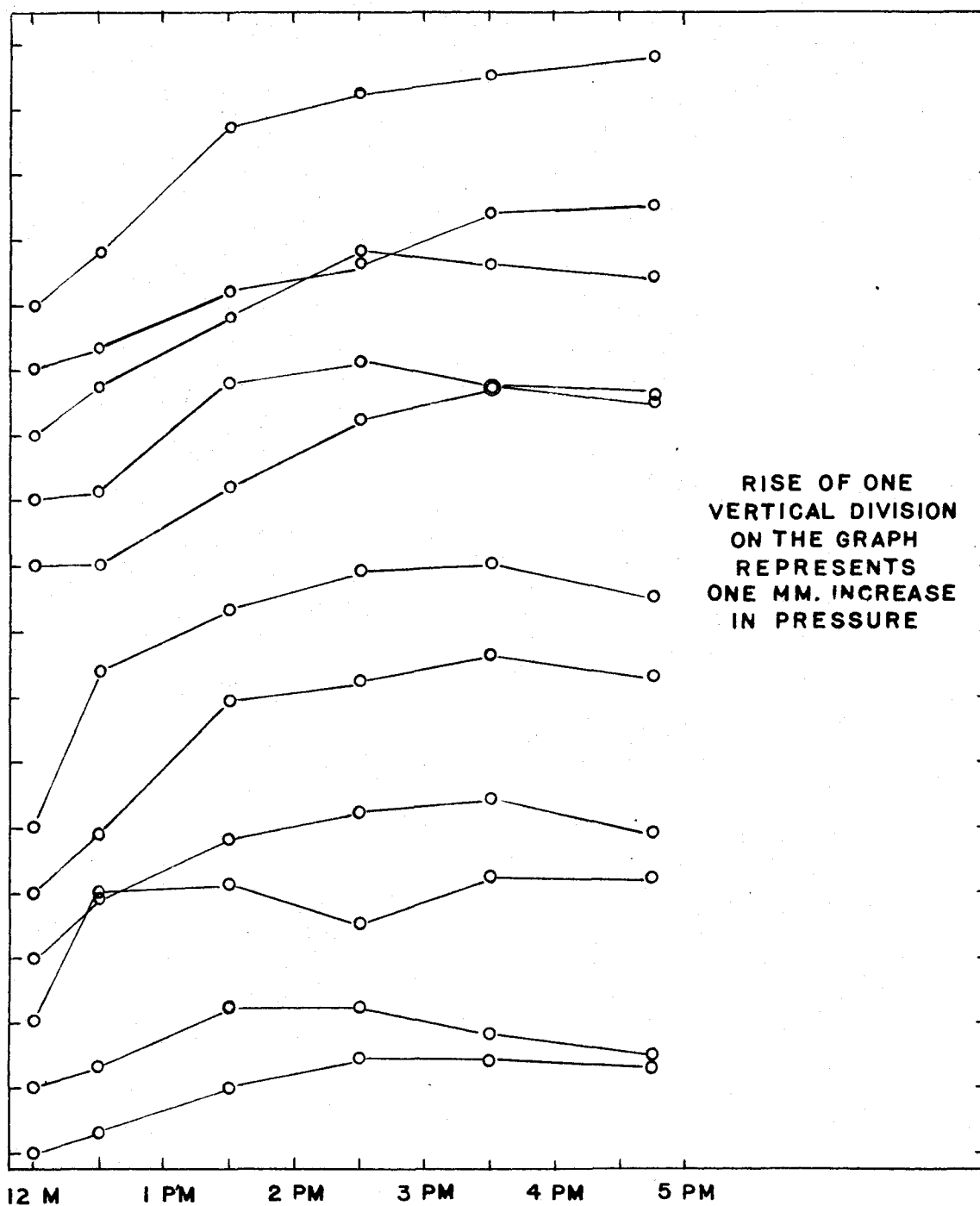


Figure 2. Pressure changes (corrected for barometric changes) in flasks having no solvent, sample or catalyst.

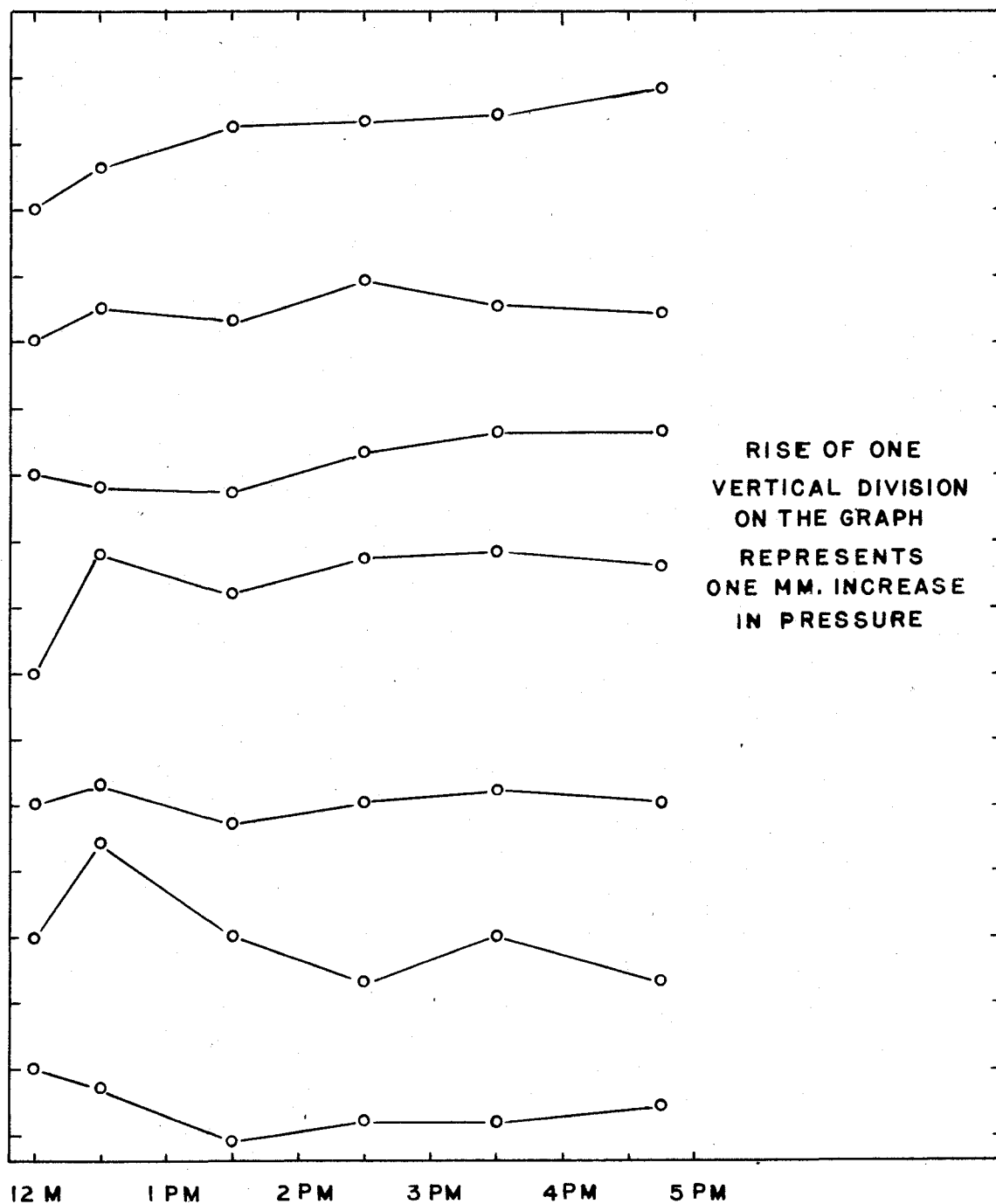


Figure 3. Pressure changes (in flasks having no sample, solvent or catalyst) corrected for changes in flasks in the usual thermobarometer positions.

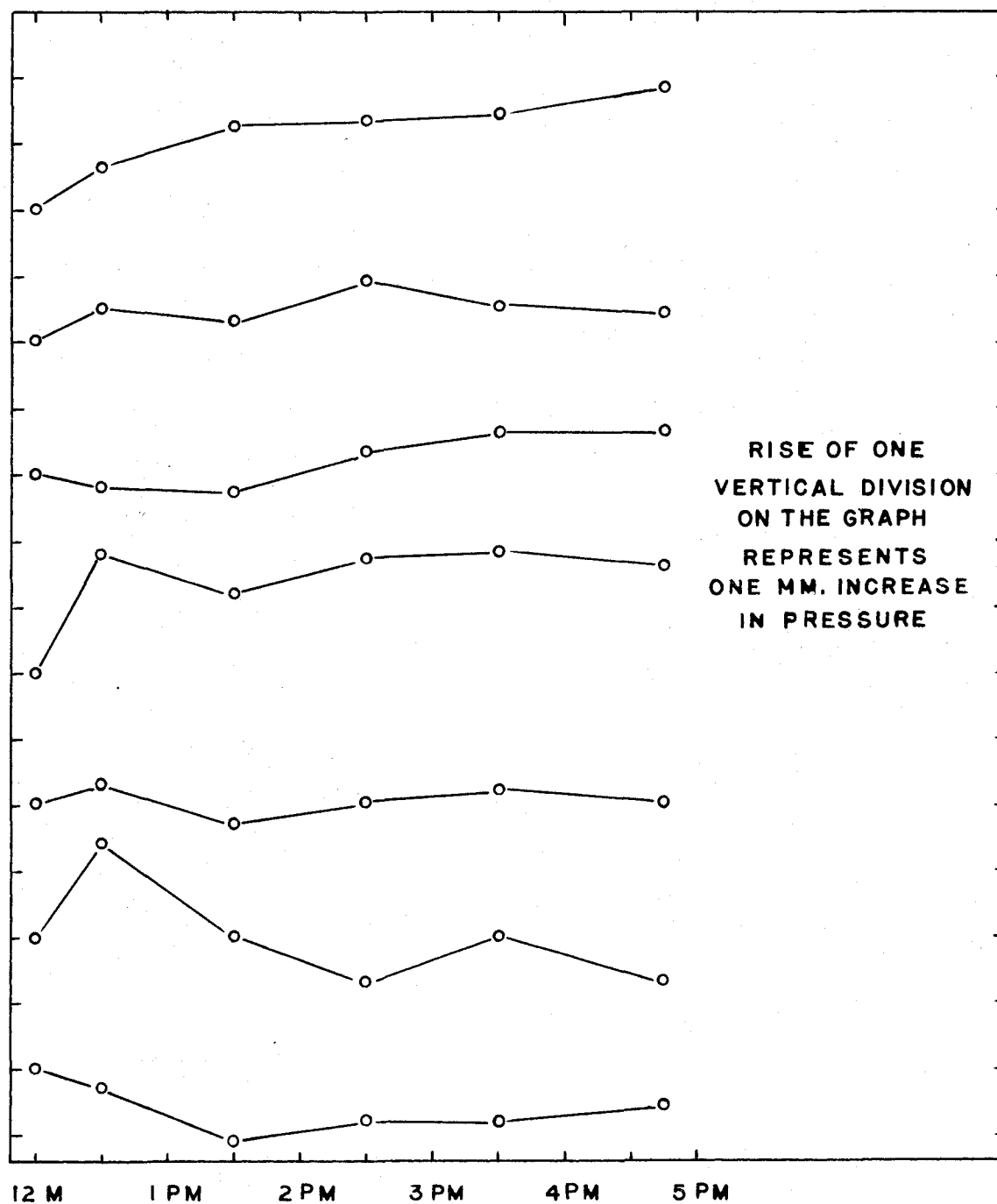


Figure 4. Pressure changes (in flasks having no sample, solvent or catalyst) corrected for average change of two flasks in thermobarometer positions.

manifold, which had been designed to allow the evacuation and filling of seven flask-manometer combinations simultaneously. Quite by accident it was noted on one occasion that hydrogen from the hydrogen reservoir had an odor much like that of rancid fat or oil. Investigation showed that all of a recent purchase of tygon tubing had the same odor and that the odor was intense. Further investigation revealed that the same odor, though generally less intense was present on all tygon tubing. As a result, an all glass hydrogenation system was developed so all parts could be thoroughly cleaned.

The development of an all glass system required a glass mercury reservoir, the volume of which could be changed at will, raising or lowering the level of the fluid in the manometers. For this purpose, Pyrex syringes were sealed to the manometers below the stopcocks previously described. To hold the plunger in place and to regulate the height of the fluid, a screw device was placed below the vertical syringe so that a common axis extended through the screw and the plunger, with the top end of the screw operating against the lower end of the plunger. On the screw is placed a stop in the form of a collar cut from copper tubing. This should be of such length as to prevent the plunger from being jammed into the end of the syringe where it has been constricted for sealing onto the manometer. A second screw at right angles to the first serves as a lock for the first.

Thus, the four ways in which the usual Warburg manometer was modified were (a) ground glass joints for attaching an evacuation and filling manifold, (b) black background and white scale, (c) all glass mercury reservoir and (d) a stop-cock between reservoir and manometer arms.

(2) Calibration. Since the space above the manometer fluid in a manometer is gas space, it must be calibrated for volume. As the first step, obtain one flask (or preferably two, as a check) and after assembling it, fill with that quantity of mercury which, when the taper joint of the manometer used in calibrating the flasks is placed on (or in) the flask, the mercury will rise to the same level in the capillary of the side arm as when the flask calibrations were made. This should preferably be that manometer which will cause the mercury to rise least. Then all other manometers are inserted in turn in the flasks and a mark made on the side arm indicating the height to which the mercury rises. This, of course, will be a different height with each manometer but should be the same regardless of which flask is used. On the balance pan place a ringstand with support rings so placed as to support the manometer in a position such as to hold mercury in the side arm and upper portion of the manometer arm proper, and obtain a tare weight on each manometer. By means of a small needle-tipped pipette with a rubber dropping bulb, place in the manometer that amount of mercury which will just fill

the space from the mark placed on the side arm earlier in the procedure to the scale mark or marks which it is anticipated will be used as setting for the manometer fluid in the closed arm during a determination. Note the temperature of the mercury used and weigh the manometer with mercury. The weight of the mercury divided by its density at the temperature used will give the volume.

Because the etched markings on the manometer scales did not compare as well as expected, these markings were checked with a cathetometer. Since the initial readings generally fall above 200 mm. and the final readings below 100 mm., the middle third was not checked in detail. Table 6 gives for each of three manometers the distance (average of three measurements) from the zero mark to the other markings at ten millimeter intervals. Corrections which should be added at each of these intervals can be calculated from Table 6. Figure 5 gives a plot of these corrections. From the plot of the data, it may be seen that the markings on these three manometers show much similarity. Since readings are only recorded in tenths of a millimeter (and even that is somewhat an estimation), certain generalizations have been made. First, because the curves of these three manometers chosen at random are so similar, the same correction is considered applicable to the other manometers purchased at the same time since they were marked with the same marking engine. Second, the subtraction

Table 6

Corrected Readings on Manometer Scales

| Marked distance, mm. from zero | Measured distance, mm. from zero on manometer number ^a | | |
|-----------------------------------|--|--------|--------|
| | 19 | 21 | 27 |
| 300 | 299.62 | 299.65 | 299.71 |
| 290 | 289.67 | 289.77 | 289.79 |
| 280 | 279.68 | 279.76 | 279.74 |
| 270 | 269.76 | 269.84 | 269.78 |
| 260 | 259.73 | 259.86 | 259.83 |
| 250 | 249.70 | 249.86 | 249.74 |
| 240 | 239.80 | 239.80 | 239.80 |
| 230 | 229.86 | 229.88 | 229.88 |
| 220 | 219.84 | 219.92 | 219.92 |
| 210 | 209.75 | 209.86 | 209.84 |
| 200 | 199.75 | 199.85 | 199.89 |
| 100 | 99.93 | 100.11 | 100.12 |
| 90 | 89.95 | 90.10 | 90.14 |
| 80 | 79.99 | 80.09 | 80.07 |
| 70 | 70.07 | 70.19 | 70.18 |
| 60 | 60.07 | 60.14 | 60.14 |
| 50 | 50.08 | 50.11 | 50.10 |
| 40 | 40.09 | 40.11 | 40.14 |
| 30 | 30.06 | 30.10 | 30.09 |
| 20 | 20.10 | 20.11 | 20.10 |
| 10 | 10.03 | 10.02 | 10.05 |

^a Each figure is an average of three readings.

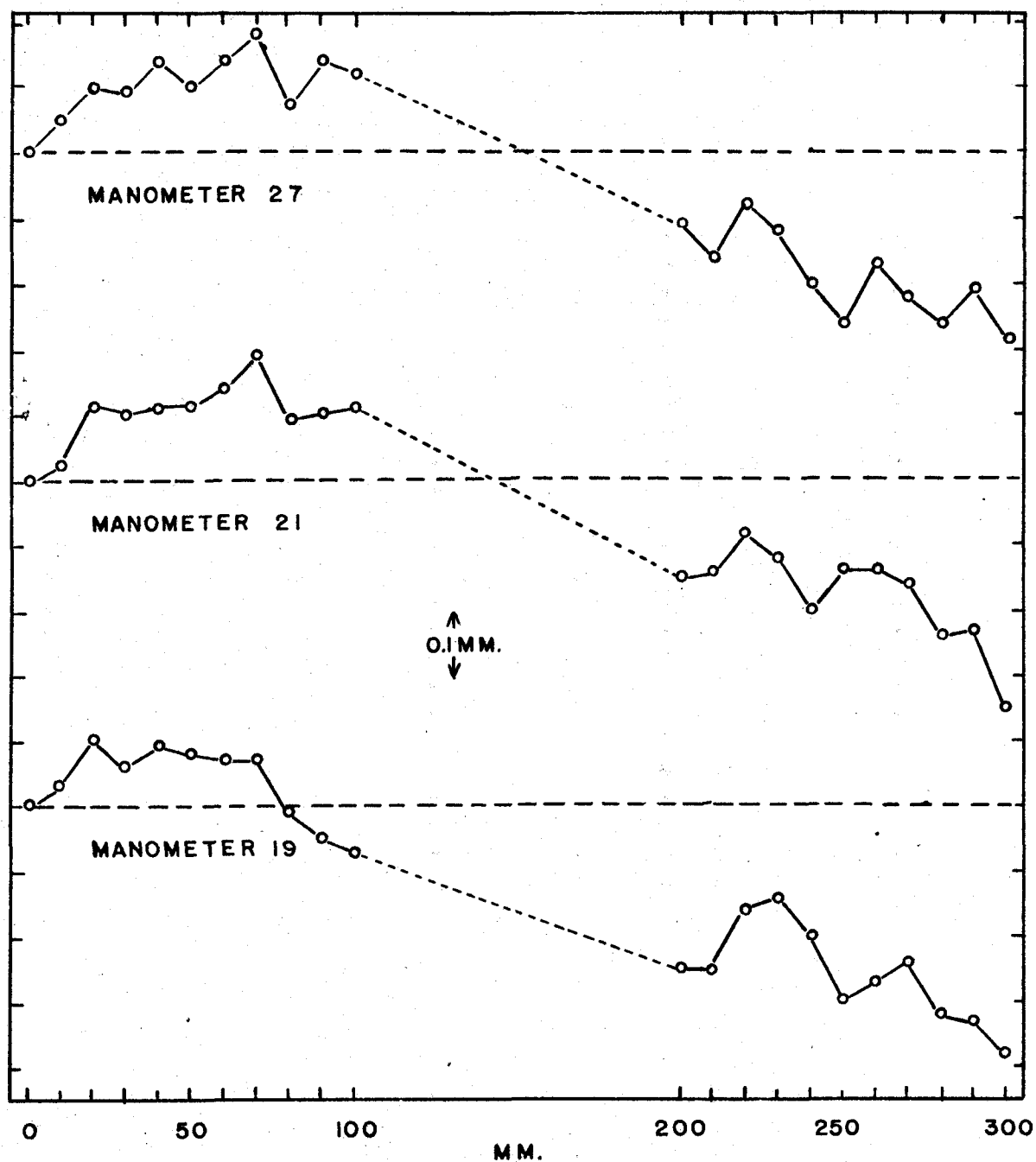


Figure 5. Deviations of manometer scale markings from true distances from zero mark.

of 0.3 mm. is considered to give a correction which is within the limits of accuracy of reading the manometers. This figure may be arrived at in various ways. It was originally determined by inspection of a plot similar to Figure 5. Subsequently, the figures given in Table 6 for the upper third of the manometers were averaged; the figures for the lower third were averaged and subtracted from the averages for the upper third. The results were 199.70, 199.71 and 199.70 mm. whereas the figures marked on the manometers would give a difference of 200.00 mm. If one assumes a change in manometer reading of 200 mm. during hydrogenation and on this basis calculates the deviation from indicated distance for each measured point on the upper third of the manometers to the point 200 mm. below it, these deviations are found to average 0.29 mm. Calculations of the same type based upon an assumed change of 180 mm. give an average deviation of 0.28 mm. (The actual deviations range from 0.15 to 0.46 mm.)

(3) Manometer fluid. Mercury was chosen as a manometer fluid because it allows a larger sample size. It will be agreed that a less dense fluid would be more sensitive to small changes in pressure. However, the design of the Warburg apparatus and the method of its use are such that the measured pressure change must be about 200 mm. in terms of the fluid used. Therefore, a less dense fluid would merely reduce the size of sample without increasing the reading accuracy. By

reducing sample size, the relative effect of certain absolute errors, as for instance sample weight would be magnified.

(4) Manometer fluid "topping". During a considerable portion of the development phase of this procedure, it was noted (a) that often the mercury column in the manometers exhibited a tendency to "stick" (i.e., not to flow freely) and (b) that there often formed in the open ends of the manometers, a white ring on the glass at the upper surface of the mercury. This ring was apparently formed of particles of lubricant carried from the stopcock by the surging action of the mercury during shaking. The explanation for its failure to form in the closed arm seems to rest in the fact that when acetic acid is used as a solvent in the reaction flasks, a certain amount of it distills into the manometer, in some cases forming a layer 2-3 mm. deep in 3-4 hours. The tendency for the mercury to stick was not particularly apparent on the first one or two runs made after cleaning the manometers. Since it was at least a two day process to disassemble, clean, dry and reassemble the manometers, it would be impossible to make regular determinations at frequent intervals on a series of fat samples if the manometers require cleaning after each second run. It was found that by topping each mercury column with about a 20 mm. column of propionic acid, these grease rings did not form and the mercury flowed even more freely than in a freshly cleaned manometer. This propionic acid did

not interfere with obtaining a proper reading since the point at issue was relative pressures or, rather, pressure changes and not absolute pressures. Any acid on the mercury column at the beginning would also be there at the end of the hydrogenation. Table 7 gives the readings obtained on a particular manometer (number 20 on April 23, 1952) without the added acid and later the same day with added acid. Three readings were taken at each interval. Also given are the barometer readings. The bath temperature during these intervals ranged from 59.98° to 60.03°. It may be noted that while the first set of readings were erratic particularly after 150 minutes, the second set, without cleaning the manometer, gave almost perfect agreement. (Note: In the second set a fat sample was hydrogenated beginning after the reading at 130 minutes.)

Further work showed that when the reading difficulties were overcome a method was developed which was suitable for quantitative hydrogenations; the erratic readings seem to have been eliminated by the use of the acid on the mercury columns.

c. Sample vials. Sample vials of various sizes and designs have been employed in this study. The most satisfactory one, which has been adopted as standard, was made from square tubing obtained from Fisher and Porter Co., Hatboro, Pa. These vials were 13 mm. by 13 mm. by 25 mm. The hole in the vial was 4-5 mm. in diameter. It should be no larger if

Table 7

Effect of Acid Column Above Mercury upon Manometer Readings

| | | | | | | | | | |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Elapsed time, minutes | 45 | 75 | 105 | 135 | 155 | 185 | 215 | 245 | 275 |
| Barometer readings, mm. | 741.1 | 741.3 | 741.5 | 741.7 | 741.4 | 741.4 | 741.5 | 741.5 | 741.4 |
| Manometer readings, mm. (without acid) | 243.1 | 243.3 | 242.9 | 242.7 | 232.0 | 232.8 | 233.8 | 233.0 | 233.0 |
| | 243.0 | 243.2 | 242.7 | 242.4 | 232.1 | 233.0 | 233.9 | 233.2 | 233.6 |
| | 243.1 | 243.4 | 242.8 | 242.4 | 232.9 | 233.8 | 234.0 | 234.0 | 233.8 |
| Elapsed time, minutes | 40 | 70 | 130 | 175 | 205 | 235 | 310 | | |
| Barometer readings, mm. | 741.2 | 741.2 | 741.2 | 741.4 | 741.8 | 741.6 | 741.7 | | |
| Manometer readings, mm. (with acid) ^a | 281.9 | 281.9 | 281.9 | 123.0 | 122.0 | 122.0 | 121.7 | | |
| | 281.9 | 281.9 | 281.9 | 122.9 | 122.0 | 122.0 | 121.8 | | |
| | 281.9 | 281.9 | 281.9 | 123.0 | 122.0 | 122.0 | 121.9 | | |

^aSample in the flask was hydrogenated after the third set of readings.

6 mm. beads are used.

d. Bath and shaker

(1) Bath fluid. Because of the high temperature used in most of these hydrogenation studies, water, the usual bath fluid in a Warburg apparatus, would evaporate too rapidly. Mineral oil was chosen as a fluid which would not evaporate and yet would have, at 60° , a low enough viscosity to permit adequate shaking without too great a strain on the manometers. It was necessary to add about 0.5 g. hydroquinone per gallon of oil to prevent oxidative deterioration.

(2) Temperature control. Since the pressure of a gas is (approximately) proportional to its absolute temperature, a change of 0.1° at 60° (or 333.17° K) will cause a change of pressure of about 0.3 parts per thousand. By interpolation from a table of acetic acid vapor pressures (119) it is estimated that the vapor pressure of acetic acid will change (in the neighborhood of 60°) about 0.4 mm. per 0.1° C. With a change in pressure of 200 mm. during hydrogenation, the 0.4 mm. is equivalent to two parts per thousand. From the equation for the vapor pressure of propionic acid (119), it is calculated that a change of temperature of 0.1° will change the vapor pressure 0.14 mm. or 0.7 parts per thousand in a total change in pressure of 200 mm.

From the above, it can be seen that with acetic acid as a solvent, a change of temperature of 0.1° can change the

results by 2.3 parts per thousand. With propionic acid the error due to change in temperature would be in the neighborhood of one part per thousand but even this emphasizes the need for good temperature control.

Since acetic acid was used during most of the developmental phase of the hydrogenation work, considerable attention was paid to temperature control. To obtain adequate temperature control in all parts of the bath, two heavy duty stirring motors were used (one motor is standard equipment with water at 37°). The original equipment was equipped with a bimetallic thermoregulator in conjunction with a relay. Even with the low current load, the contact points quickly pitted and temperature control became poor. A merc-to-merc thermoregulator was tried in conjunction with a relay but without much success, due mainly to the lag but also in part to occasional failure of the mercury surfaces to make a current carrying contact (apparently due to a bubble of gas).

In an attempt to reduce the lag on the merc-to-merc thermoregulator, a specially shaped coil immersion heater and other flexible immersion heaters were ordered but were not received in time for use. It had been planned to use continuous heaters, with capacity to almost but not quite keep the bath at proper temperature. The specially shaped heater was to be used as an intermittent heater with the thermoregulator in the center of the heating coil and the two of them so

placed that there would be very rapid circulation of the bath fluid past them.

In lieu of adequate temperature control by the usual thermoregulators, the temperature was controlled by means of a variable transformer in series with a 300 watt immersion heater. While there was considerable fluctuation in line voltage, a heater rated at 300 watts at 115 volts would, on direct connection, just about maintain proper temperature. As long as line voltage remained constant, the temperature could be controlled within very narrow limits by means of the transformer, on occasion, varying less than 0.2° in half an hour.

Temperatures were measured by means of a differential thermometer having a range of five Centigrade degrees. After setting for the desired range, the mark corresponding to 60° was located by checking with a thermometer certified by the National Bureau of Standards. The differential thermometer could easily be read to one hundredth of a degree, its smallest calibration interval.

(3) Shaking. The manometers and attached flasks were shaken at a rate of 120 cycles per minute through a four centimeter stroke. To increase the effectiveness of the agitation, each flask contained ten glass beads of 6 mm. diameter.

e. Use of apparatus, general

(1) Solvent. During most of the development of this hydrogenation method, acetic acid was used as a solvent.

Dedrick and Bird (43) had found that the hydrogenation did not proceed smoothly in a non-polar solvent. The acetic acid used was redistilled and checked for reducing substances (186). Acetic acid was satisfactory from the points of view of dissolving all the fat and allowing the reaction to proceed smoothly. However, during the determinations, a small amount of the acid distilled over into the manometers forming a column of acid on top of the mercury column in the closed arm of the manometer, and thus affecting the readings. In order to avoid this difficulty, a change was made to propionic acid. With this acid there was no visible evidence of distilling. While the procedure as finally adopted included the use of a column of acid on top of the mercury column, this does not interfere with the reading so long as the amount of acid remains constant.

(2) Reading. There were indications that manometer readings (in the early phases of development) might not be consistent with pressure changes. As a result, six methods were compared. These were:

(a) Adjust all manometers on one side. (This major adjustment consisted of raising the mercury above the usual mark, lowering it below the mark and slowly bringing it to the mark.) Begin reading the first manometer one minute after adjusting it with minor adjustments if necessary and proceed to all others on the same side. Readjust all and then

immediately read all. Again readjust all and read all a third time.

(b) This method was similar to method (a) except that there were three one minute waiting periods, one before each reading.

(c) Same as procedure (a) except with a two minute delay.

(d) Same as procedure (a) except that the one minute waiting period is after shaking has stopped but before the adjustment and each manometer is read immediately upon adjustment.

(e) In this procedure, each manometer is, in its turn, adjusted and read immediately, the only waiting is that between readings and is that necessary to read other manometers.

(f) This procedure is one that is only applicable to situations where manometers on both sides are to be read. In this all are adjusted and one minute after adjusting the first one, reading is started. As soon as those on one side have been read, they are readjusted, after which those on the other side are read and readjusted. This is continued until each has been read three times.

The procedure finally adopted was that listed above under

(d) wherein upon stopping the shaker, the manometers remain quiet for one minute, after which each manometer in turn is adjusted and immediately read. This is then followed by two

more readings.

It was arbitrarily decided that if the highest and lowest of the three readings differed by not more than 0.4 mm., the average of all three would be used. If the range was greater than this but two fell in the range, the average of these would be used, while if no two were within 0.4 mm., then all would be discarded. The average at the first reading is then subtracted from each subsequent average and the resulting figure (with proper sign) recorded as ΔH . The ΔH for the thermobarometer (or their average if more than one is being used) is subtracted from each ΔH for a manometer with sample and the resulting figure (with proper sign) is recorded as $\Delta H'$. If three of these last figures are in substantial agreement the pressure is considered constant.

(3) Correction of reading. Since a Warburg manometer measures pressure relative to atmospheric, any change in barometric pressure will affect the reading, as will likewise a change in bath temperature. Correction for these factors should be obtainable by the use of thermobarometers identical with the sample units except that no catalyst is added. As long as the bath temperature is constant, the changes in the thermobarometers should be equal in magnitude but opposite in direction to those of the barometer. That this was not the case was noted throughout most of the development phase.

Figure 2 on page 106 shows the results of eleven flasks set up

without sample, solvent or catalyst. The temperature was so nearly constant that these results should have plotted as straight lines.

Five possible causes of these results were considered.

(a) Something in the system is giving off an absorbed gas.

(b) Something in the system is volatilizing, possibly with decomposition.

(c) The gas system is extremely slow in reaching a temperature equilibrium.

(d) The temperature, and hence the density of the manometer fluid changes.

(e) The mercury does not flow freely in the manometers.

(Note: The reader is again reminded that the material presented in this portion of the thesis is grouped according to processes and equipment and is not chronologically arranged except within these groups mentioned.)

A thermometer placed beside the mercury column in one of the manometers failed to show any measurable change in temperature, thus eliminating (d) as a possible cause.

If the system were slow in reaching a temperature equilibrium, then heating to a higher temperature and allowing the system to approach the desired temperature from above should either eliminate or reverse the trend. The results of heating to 65° for 30 minutes and then allowing the system to

cool to 60° are shown in Figures 6 (uncorrected) and 7 (corrected for barometric changes). During the period of taking these readings the temperature covered a range of 0.4° . From these it may be seen that the trends are of the same type as found when approaching 60° from below (Figure 1), which seems to eliminate (c) above.

It was after extensive work with flasks containing no catalyst and in many cases no sample or solvent but giving results very similar to those shown in Figures 1 to 5 that a foreign odor from the hydrogen supply reservoir was noted. To eliminate possible compounds associated with this odor in the water-pumped hydrogen, an all glass hydrogen generator and washing train was constructed. The hydrogen was freed of oxygen by washing with sodium stannite solution, and of sulfur and halogens by silver nitrate, was dried with indicating drierite and P_2O_5 and finally by passage through a liquid nitrogen trap. Results with hydrogen so prepared, with properly cleaned glassware and with manometers in which propionic acid was placed on the mercury columns are shown in Figure 8. These data indicate that pressures before and after the samples were tipped were constant.

The reason for the pressure increase on dissolving of fat in solvent is not known. It is thought that hydrogen dissolved in fat may be released when the fat is dissolved in propionic acid or vice versa. Nothing has been found in the literature

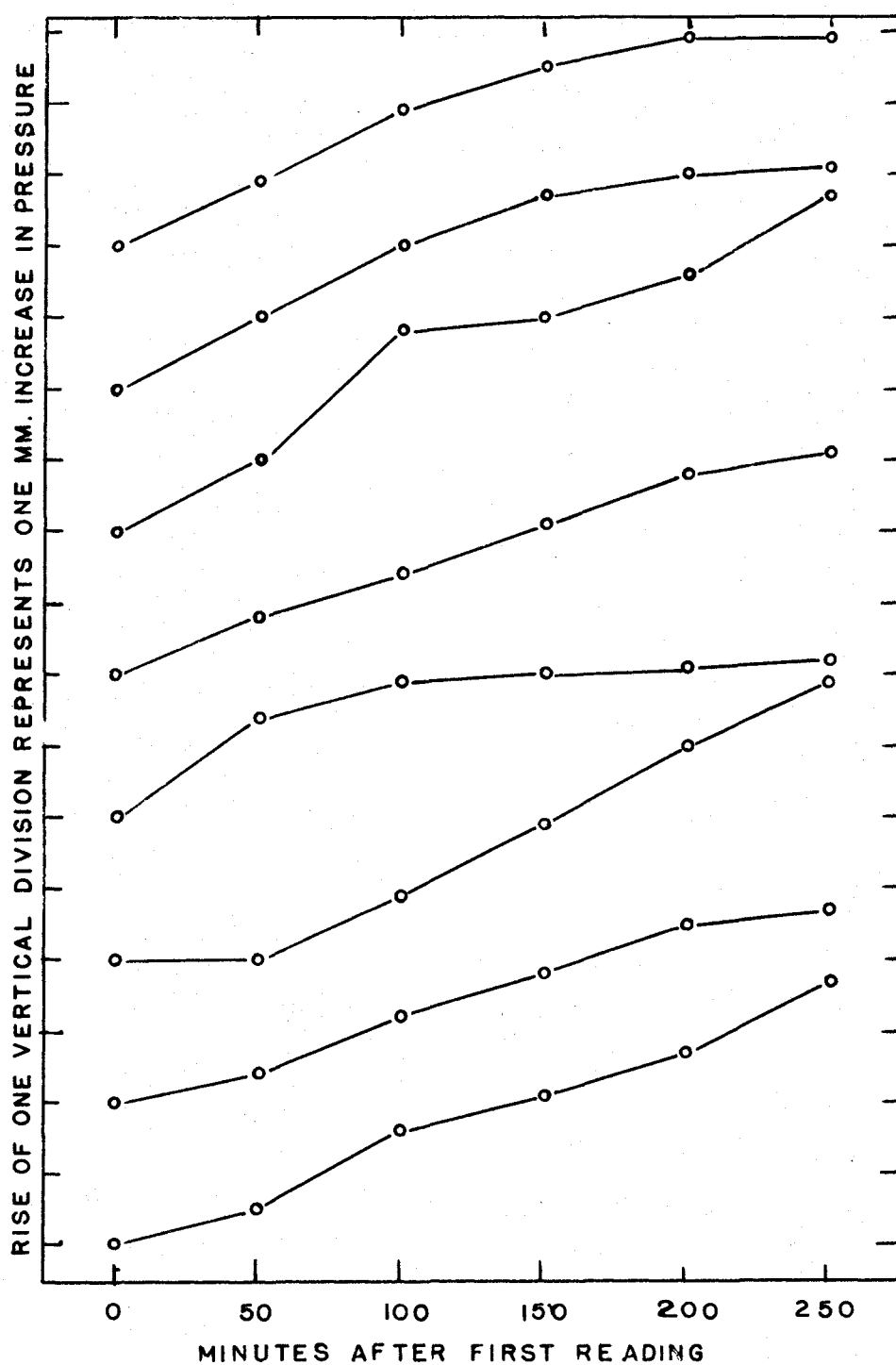


Figure 6. Pressure changes (uncorrected) in flasks at 60° after initially heating to 65° for 30 minutes.

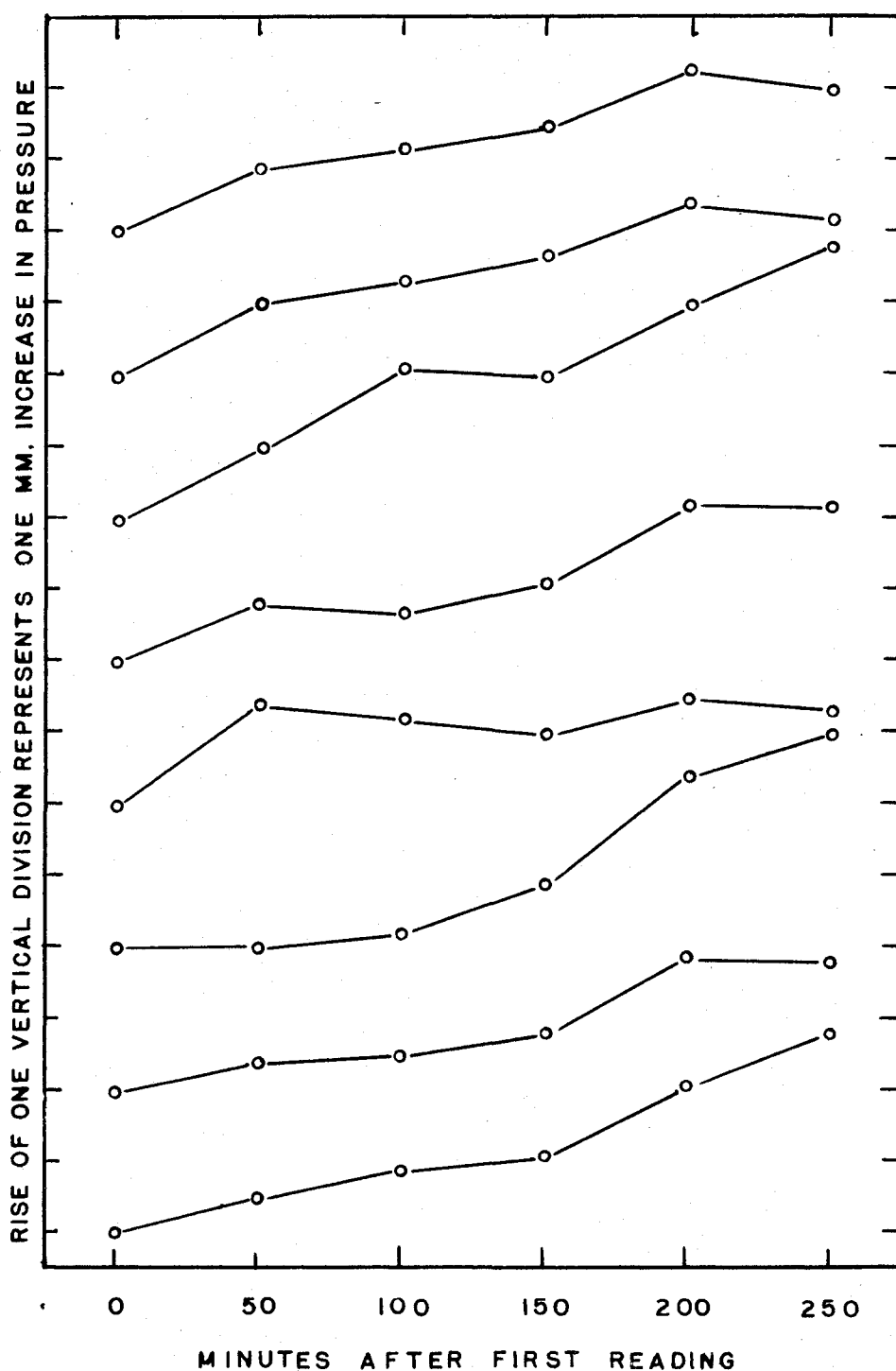


Figure 7. Pressure changes (corrected for barometric changes) in flasks at 60° after initially heating to 65° for 30 minutes.

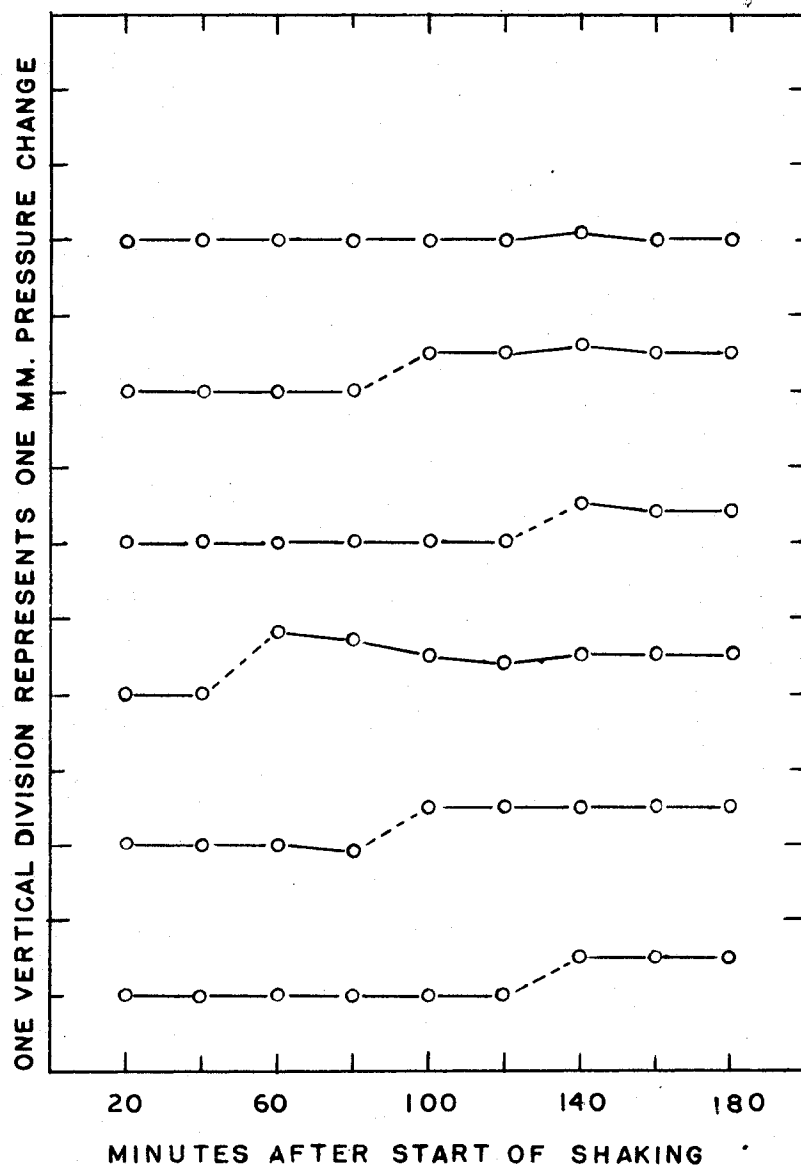


Figure 8. Pressure changes in hydrogenation flasks. Broken lines indicate interval during which fat was tipped from sidearm into solvent.

concerning solubility of hydrogen in either propionic acid or milk fat, or in mixtures of the two. However, since the pressure increase is always present, the best that can be done is to include fat in the thermobarometer with the expectation that the increase in each reaction flask will be the same as that in the accompanying thermobarometer. Some indication that this may not be true is shown by the data in Table 8 which presents the pressure increases corrected for barometric changes in thermobarometers in the study reported subsequently in this thesis. Zaletel and Bird (218) found it impossible to correlate the weight of catalyst and the amount of hydrogen uptake. Therefore a thermobarometer containing catalyst but no sample would be unsatisfactory.

(4) Cleaning of glassware. Unclean glassware can affect the results of chemical procedures. Cleaning glassware in an oxidizing acid bath has been accepted as a standard procedure. In the beginning the cleaning was done with a hot mixture of about 19 parts H_2SO_4 to one part HNO_3 , after cleaning with a brush and detergent mixture. Frequently glassware was judged to be unclean because the surface would not drain without droplets adhering, when cleaned as described above. Various cleaning procedures, using organic solvents, synthetic detergents and acid mixtures were found unsatisfactory.

Table 8

Effect of Dissolving Fat in Propionic
Acid on Pressure in Thermobarometers^a

| Date, July | 30 min. prior to tipping | Immediately prior to tipping | 45 min. after tipping | 75 min. after tipping |
|---------------|--------------------------------|------------------------------------|-----------------------------|-----------------------------|
| 2 | - 0.3 | - 0.2 | + 0.9 | + 0.8 |
| 8 | + 0.7 | + 0.5 | + 1.7 | + 1.5 |
| 13 | + 0.1 | + 0.2 | + 1.8 | + 1.6 |
| 17 | + 0.8 | + 1.0 | + 2.8 | + 3.0 |
| 22 | + 0.6 | + 0.6 | + 1.1 | + 1.3 |
| 23 | + 0.3 | + 0.3 | + 1.6 | + 1.6 |
| 25 | + 0.8 | + 0.7 | + 2.3 | + 2.1 |
| 28 | + 0.4 | + 0.3 | + 1.2 | + 1.4 |
| 29 | + 0.4 | + 0.4 | + 1.6 | + 1.5 |

^aThe two readings immediately preceding tipping and the two readings immediately following tipping for all cases in July, 1952 in which sufficient barometer readings were taken to allow correction for barometric change. Readings expressed as mm. change (after barometric correction) from first thermobarometer reading. In all cases temperature range was less than 0.05° during the 105 minute period included above.

On the assumption that a certain amount of acid might be absorbed on the glass and not rinsed off by water, a five minute soak in dilute (about 2 N.) NH_4OH was tried without apparent effect. Concentrated (about 14 N.) NH_4OH yielded no better result; and the same was true of a one hour soak in conc. NH_4OH . However, an eight to twelve hour soaking in 2 N. NH_4OH after a combination detergent wash-hot acid soak cleaning was found to be effective. In order to be sure all

NH_4OH is removed from the glassware, it was dried in an oven at 200° for two hours.

Large particles of silicone grease were effectively removed by turpentine, which can be removed by a synthetic detergent.

The cleaning procedure adopted for the glassware is as follows:

(a) Hydrogenation flasks. Wipe the outside of the flask and manometer side arm with a towel, cheesecloth, cleansing tissue or similar material to remove as much mineral oil as possible. Wipe around the flask-manometer joint with facial tissue wet with ethyl ether. Remove flask from manometer and wipe the ground portion of the manometer joint with facial tissue wet with ether. Regrease this joint and cap it with a sealed off standard-taper joint. Remove clamps or springs from flasks and again wipe the latter with a cloth or tissue. Without opening flask place it in a beaker (600-800 ml.) containing enough ether (wash ether No. 1) to almost completely immerse the flask. After about a minute remove flask and open it. Wipe all ground joints, first with dry tissue and then with tissue wet with ether. Wash fat, propionic acid and catalyst from flasks and vials with a stream of hot water, putting vials and beads in a beaker. Wash the flasks in a synthetic detergent solution, brushing all surfaces inside and out. Rinse, invert to drain and then

dry with towel or cheesecloth. Immerse in a second beaker of ether (wash ether No. 2), completely filling and immersing flask. (Note: From time to time it will be necessary to add more ether. Usually this is best done by replenishing the supply of wash ether No. 1 from wash ether No. 2 and adding fresh solvent to the supply of wash ether No. 2. Eventually it will be necessary to discard completely the wash ether No. 1.). Lids to hydrogenation flasks need not be washed with detergent until after rinsing in wash ether No. 2, and need not be wiped. Place flasks (including lids in hot mixture of nitric and sulfuric acids for 30 minutes. Remove from bath and allow to cool under fume hood. Rinse with tap water three times and distilled water once. Immerse in 1.5-2.0 N NH_4OH for eight hours or more. Remove from NH_4OH solution, rinse at least three times in distilled water and dry in 200° oven for two hours.

(b) Catalyst vials, sample vials and beads.
Clean as thoroughly as possible with a stream of hot water. Place in a beaker of synthetic detergent solution and shake thoroughly for about a minute. Rinse with tap water and place in a small jar with ethyl alcohol. Place lid on jar and shake for about 30 seconds. After removing alcohol put the glassware through two ether washes in a similar manner. Spread the vials out in a glass dish (or shallow beaker) being sure vial openings are not on the bottom (preferably on the side) and

dry on steam plate or low heat hot plate. Put glassware into mixture of nitric and sulfuric acids in a beaker (1000 ml.) and heat, being sure all vials are filled with acid. Turn off heat after bath has been hot (100° or more) for 30 minutes. When the acid has cooled sufficiently, decant (into another beaker) as much acid as possible using a watch glass to hold back vials and beads. Rinse (cautiously) several times in beaker with tap water, followed by distilled water. Using forceps, pick up each vial individually and rinse the inside with distilled water. This can best be done by inserting into the end of the distilled water line a piece of glass tubing drawn down to give a fine stream. Soak vials and beads in 1.5-2.0 NH_4OH for at least eight hours. Pour off NH_4OH solution, rinse thoroughly, inside and out, with distilled water and dry in a 200° oven for two hours.

(c) Manometers. With a very fine capillary pipet and a rubber bulb, remove as much as possible of the propionic acid from the surface of the mercury. Invert the manometer so the mercury will drain out of the normally open arm and after opening stopcock adjacent to the syringe, work plunger back and forth until all mercury is out of the manometer. Remove stopcock plugs from manometers, being sure they are numbered to correspond with the manometer numbers. Remove manometers from supports, keeping all small parts for each support in a separate container. Remove plungers from

syringes being positive these are marked, as they are definitely not interchangeable. Wipe all ground surfaces, first with a dry tissue, then with a tissue wet with ether. Grease can be removed from the bore of stopcock plugs with a pipe cleaner. Strip the black masking tape from the manometers and soak the manometers in turpentine for 20-30 minutes to remove any particles of silicone grease from inside the capillary bore of the manometers. Rinse thoroughly in a synthetic detergent solution and if any paint remains in the etched scale markings, soak in a cylinder of concentrated NaOH for a sufficient time to loosen the paint so it can be rubbed off. Rinse successively with water, alcohol, ether, alcohol and water. This is best done by using a tube bent into the form of a "J" with the outer portion of a standard taper joint on the short end and with the long end longer than the manometers. Place a plug in the stopcock adjacent to the syringe and invert the manometer into the ground joint of the "J" tube. Pour the liquid into the long end of the "J" tube, the syringe and the sidearm of the manometer. Place the manometer into a hot mixture of nitric and sulfuric acids, with all parts being submerged for at least 30 minutes. It will probably be necessary to do first one end and then the other. Rinse thoroughly with water and soak in 1.5-2.0 N NH_4OH for at least eight hours. Rinse thoroughly with distilled water and dry in a 200° oven for two hours. After drying, spread white

paint over scale markings and wipe off excess with cleansing tissue. Allow to dry and place strip ($3/8$ inch wide) of black masking tape on back of manometer arms. Grease and replace stopcocks and secure them in place (rubber bands will do). After greasing syringe plunger, invert manometer (with stopcocks closed) and place about 1.5 to 2.0 ml. mercury in syringe. Insert tip of plunger into syringe just sufficiently to close the end (do not force). While holding syringe in place turn manometer upright, open lower stopcock and force air out of syringe. Continue with reassembling manometer.

(d) Other parts. Dismantle entirely, removing plugs from stopcocks. Using a dry cleansing tissue, wipe off as much grease as possible. Wipe again on ground joints using cleansing tissue wet with ether. In some cases a pipe cleaner will be helpful in removing grease from the inside of small parts. For parts which cannot be effectively wiped clean, soak in turpentine for 20-30 minutes. Wash in synthetic detergent solution, brushing wherever possible. Rinse with water and soak in hot acid bath 30 minutes. Rinse with distilled water and soak at least eight hours in 1.5-2.0 N NH_4OH . Rinse in distilled water and dry two hours at 200° .

3. Other equipment and its use

a. Hydrogen generation. Originally, compressed hydrogen, water pumped into cylinders, was used. With the thought that

possibly this might be a factor in the inability to obtain equilibrium pressures, this was discontinued. Instead, hydrogen was generated from 30 mesh reagent grade zinc (low in lead, arsenic and iron) and sulfuric acid (special reagent grade, low in arsenic and nitrogen).

The main body of the hydrogen generator consisted of a three-liter Erlenmeyer flask, to the neck of which had been sealed the outer portion of a 29/42 standard taper joint. Into this was fitted the inner portion of the joint above which a cylindrical 500 ml. separatory funnel with a dropping tip was sealed. A pressure equalizing tube connected the upper portion of the dropping funnel with the tube below the stopcock. Above the funnel, mounted with a ground joint was a combination manometer and mercury sealed pressure relief valve.

The hydrogen storage system consisted of two five gallon bottles, one set 30 inches above the other, with a glass tube connecting the two and extending nearly to the bottom of each. The entire system was constructed of glass with ground joints. In the beginning, the lower bottle was filled with redistilled water (the second distillation from acid dichromate in an all glass system). As hydrogen was generated, it entered the lower bottle, forcing water into the upper bottle, affording a hydrostatic head on the reservoir.

b. Hydrogen purification. When commercial hydrogen was being used, it was bubbled through an alkaline sodium stannite solution to remove oxygen. This solution was made up as follows: Dissolve 50 g. NaOH in 200 ml. H_2O . In another flask, dissolve 10 g. $SnCl_2$ in 50 ml. H_2O . Add the NaOH solution to the $SnCl_2$ solution with constant stirring. Allow to stand several days and decant solution from any precipitate.

Following the stannite solution treatment the hydrogen was dried with indicating drierite (anhydrous $CaSO_4$ coated with $CoCl_2$) and then with P_2O_5 .

When hydrogen was generated from zinc and sulfuric acid, it was passed through a 1 N $AgNO_3$ solution to remove any volatile sulfides and halogens that might be present. This was in addition to the purification steps previously used. As a final step the hydrogen was passed through a trap immersed in liquid nitrogen.

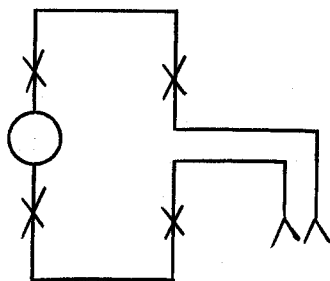
For the hydrogen purification, gas washing tubes were designed and constructed to include a built in trap to prevent the liquid from being backwashed into the line or to other parts of the apparatus. The body of these tubes made of tubing about 40 mm. diameter sealed off with a test tube-like bottom and having at the top the outer portion of a 29/42 standard taper joint. Just below this joint was a small side tubulation bearing the socket part of a ball and socket joint. The other portion of the washing tube had a piece of tubing

about 22 mm. diameter sealed to the small end of the inner portion of the 29/42 ground joint. To the lower end of this was sealed a gas dispersion tube with a fritted cylinder. Above the inner portion of the 29/42 joint is sealed the outer portion of another standard taper joint, usually smaller but still large enough to allow the brushing of the inside of this inner tube when necessary. Into this joint fits a cap-like piece bearing one portion of a ball and socket joint. As the gas comes into the central portion of this gas washing tube, all liquid is forced into the peripheral space and the hydrogen bubbles through it there. In the event of a reversal of flow, liquid is forced into the central tube and the gas bubbles through it there without carrying liquid to other parts of the apparatus.

c. Evacuation and filling. To remove any condensible material, particularly any coming from the vacuum pump, two traps immersed in liquid nitrogen are employed. These must be immersed in the nitrogen before the vacuum pump is connected but must not be left open to the atmosphere any appreciable time after being cool, because if they are left open oxygen will condense in the tubes and make it difficult to obtain a vacuum. Once the traps have been connected to the pump, they must not be allowed to warm up until they have been disconnected, and then must be cleaned before reuse.

After the samples, catalyst and solvent have been placed in the flasks, the flasks are closed and secured with either springs or clamps, depending on the type of flask. After placing the flasks on the manometers, the mercury columns are adjusted to approximately the 200 mm. mark and the stopcocks are properly set.

Since flexible tubing could not be properly cleaned, it was necessary to devise glass connections for evacuating and filling the flask-manometer combinations. To be able to handle several combinations simultaneously a manifold was designed. A cross-section of the manifold and connecting tubes is shown diagrammatically below.



The large circle represents the manifold and the straight lines the connecting tubes. The X's represent ball and socket joints while the inverted V's indicate standard taper joints fitting onto the manometers.

In order to be able to remove all dissolved oxygen, it is necessary that the fat be liquid at the time of evacuation. For this purpose, a small electric clothes pressing iron has

been found to be a convenient portable hot plate which can be held against the under side of the flask sidearms. Heat should be applied only long enough to melt the fat.

Air is removed from the system by evacuating to 10 mm. pressure and holding for 10 minutes during the first evacuation. Hydrogen is then admitted to the system, passing through only one of the cold traps, by-passing the cold trap nearest the vacuum pump. After raising the hydrogen pressure to within about 100 mm. of atmospheric pressure (as indicated on an open end manometer), the system is again evacuated; as soon as a pressure of 10 mm. or less is reached, hydrogen is admitted. This procedure is continued to a fourth evacuation. On the fourth filling the thermobarometers are filled to within 50-60 mm. of atmospheric pressure while the determination flasks are filled to about 15 mm. less than atmospheric pressure. After properly adjusting the stopcocks and disconnecting the manifold, the taper joints at the manometer tops should be capped with pieces of glass tubing bearing taper joints. The purpose of these caps is to reduce the possibility of mineral oil getting into the manometers or onto the ground joints. The caps on the open ends of the Warburg manometers must have small holes in their upper extremities.

4. Catalyst

a. Preparation. In the beginning the catalyst used was a platinum oxide commonly known as Adams catalyst, prepared essentially according to the directions of Voorhees and Adams (381). This catalyst did not stay well dispersed, showing a considerable tendency to aggregate. On the hypothesis that some of these aggregates might contain unreduced oxide which would absorb additional hydrogen if the aggregates were broken up during agitation, thus accounting for the lack of equilibration, it was decided to try dispersing the catalyst on an inert carrier. Celite, a 300 mesh diatomaceous earth, was chosen as the carrier. Celite was mixed with the precipitated (and dried) ammonium chloroplatinate and sodium nitrate and the quantity of sodium nitrate was increased. By this procedure it was possible generally to obtain a catalyst of good activity. In view of the work by Frampton et al. (384) a thermocouple enclosed in a stirring rod was used to measure temperatures during the preparation of the catalyst mixtures. It was, however, almost impossible to control temperatures with this mixture over a flame. Under good conditions a catalyst made with 1 per cent platinum oxide (based on 100 per cent recovery of platinum) was found to be very satisfactory.

In order to better control the conditions, a furnace with temperature controlled at 540° was tried and found to give

good results, since it applied controlled heat to all sides. A catalyst prepared in this manner is easily dispersed and shows no tendency to form lumps.

The procedure recommended for preparation of the catalyst is as follows:

Obtain small piece of platinum and weigh. Wash platinum free of base metals by boiling in nitric acid. Remove platinum from nitric acid and dissolve by heating in aqua regia (3 HCl : 1 HNO₃), approximately 100 ml. per gram of platinum, adding more HCl as needed to keep volume constant. Filter through a sintered glass filter. To the filtrate, add slowly an equal volume of 10 per cent NH₄Cl to precipitate the platinum as (NH₄)₂PtCl₆. Filter precipitate with sintered glass filter, wash with two 50 ml. portions 10 per cent NH₄Cl. Dry the precipitate as well as possible with suction. Redissolve precipitate in aqua regia, filter, precipitate as (NH₄)₂PtCl₆, filter the precipitate, wash and dry as before. Again redissolve in aqua regia and filter. Make to a volume such that 100 ml. solution is equivalent to 1 g. of platinum.

Weigh out 12.5 g. Celite into a 400 ml. beaker. Add 100 ml. aqua regia. Stir well with an electric stirrer under a hood for 5 minutes to thoroughly disperse the Celite. While still stirring, add dropwise 10 ml. of the platinum solution. Continue stirring and add as rapidly as possible 50 ml. of 20

per cent NH_4Cl . Stir an additional 15 minutes and allow to settle.

Filter with a sintered glass filter, decanting supernatant through filter first. If possible, use a filter large enough to collect all of the precipitate at one time. Wash precipitate with two 100 ml. portions of 10 per cent NH_4Cl . Dry as well as possible by suction and transfer to a 250 ml. Vycor beaker. Add 125 g. NaNO_3 which has been ground in a glass mortar to about 20 mesh. Mix thoroughly. Dry on a steam plate or in a 110° oven overnight.

Place a pyrex glass baking dish in the muffle furnace (in case the fusion mixture "foams" over edge of beaker) and heat to 540° . When furnace is hot, set beaker of mixture, covered with pyrex watch glass, into the dish in oven and continue heat for 60 minutes. In the meantime, place about $2\frac{1}{2}$ to 3 liters of distilled water in a 4 liter beaker and set up an electric stirrer to agitate the water. At the end of the heating period, remove (with beaker tongs and gloves) the beaker containing the fusion mixture. While it is still hot, pour very slowly into the water (with stirrer running). Some splattering may take place but this, while undesirable from the standpoint of slight loss of catalyst, is not serious. After pouring into the water as much of the fusion mixture as possible, cautiously add water to that portion remaining in the Vycor beaker and wash it into the large beaker. Stir for

at least 30 minutes, longer if there are large particles or lumps. Let the material settle and decant the supernatant. Add water, stir, allow to settle and decant a second and a third time, the last time leaving about one liter of water.

Filter with sintered glass. Wash by running 500 ml. distilled water over the catalyst. This can easily be done by filling a 500 ml. volumetric flask and supporting it in an inverted position with the neck sticking down into the filter. This procedure may also be useful in the actual filtration. After the catalyst has been washed, dry as much as possible by suction. Then suck about 50 ml. of alcohol through the catalyst followed by 50 ml. ether. Remove the suction just as soon as the layer of free ether reaches the bottom of the catalyst since excessive suction will evaporate the ether very rapidly and cause cooling to the extent that moisture will condense on the catalyst and thus defeat the purpose of the alcohol and ether washes.

Transfer to a beaker and dry on a hot plate at about 100° (steam plate if possible). This drying procedure should give a finely divided precipitate which will not need grinding in a mortar.

The quantities listed here would give, if there had been 100 per cent recovery of the platinum, a preparation containing 1 per cent PtO_2 .

b. Amount of catalyst. A comparison of the amounts of Adams catalyst (no carrier) revealed that there was, even for as long as 200 minutes after hydrogenation, a continuing uptake of hydrogen. There were, however, appreciable differences in the time necessary to reach a leveling out period. They were

50 mg. - 10 min.
 25 mg. - 30 min.
 12½mg. - 40 min.
 6½mg. - 70 min.

With the catalyst on a carrier, using 9 per cent and 3 per cent preparations, results were variable with 100 mg. or less, in some cases being complete in 30-45 minutes but often taking 100 minutes or longer. With 150 mg. the hydrogen uptake practically ceased after 45 minutes or less with the values agreeing about as well as might be expected in view of other difficulties.

After changing to a 1 per cent catalyst preparation, it was found that 50 mg. catalyst required 160 minutes to hydrogenate a fat, while on the following day the hydrogenation was not complete in 360 minutes. In the evening of this second day, with the same fat and 150 mg. of catalyst, the hydrogenation was complete within 20 minutes. In the study reported with regard to fat oxidation in the latter part of this thesis, 150 mg. of 1 per cent catalyst were used and it was very

unusual for a hydrogenation to be incomplete when the first reading after start of hydrogenation was made in 45 minutes.

c. Fat as a promoter. Although it was not tested after the hydrogenation method reached the stage of attaining good equilibration of pressures (and hence precision), preliminary observations seemed to indicate that the addition to the catalyst of a few drops of fat during the equilibration period had an activating influence on the catalyst. Equilibrium pressures were apparently reached more quickly both before and after the actual hydrogenating when a few drops of fat were added to the catalyst.

d. Use of catalyst. When the catalyst was placed directly onto the bottoms of the reaction flasks, it was observed to be reduced in many cases before shaking was begun. Since it was feared that reduction under these conditions might result in a greater degree of lumping or aggregation, the catalyst was placed in a deep vial and covered with a portion of the solvent used in the flasks.

The catalyst (150 mg. \pm 1 mg.) was weighed into flat bottomed vials about 10 mm. in diameter and 25 mm. in height, which were carefully placed upright on the bottoms of the reaction flasks, against the side of the flasks. With the proper amount of propionic acid for a flask measured into a pipette, the flask was tipped over (being careful not to spill the sample or upset the catalyst vial). A portion of the

solvent from the pipette was allowed to run slowly down the side of the catalyst vial in a manner to trap as little air as possible. As soon as all the catalyst was wet, the flask was set upright and the vial filled to within about 6 or 8 mm. of the top, the remainder of the solvent being placed in the flask itself. After adding one or two drops of fat to the solvent in the flask, the flask was closed and the lid secured. After the flasks are filled with hydrogen and just before placing them in the Warburg bath, the catalyst vials are tipped over.

5. Calculation of hydrogenation iodine values

The equation for converting manometer readings to hydrogenation iodine values is as follows:

$$\frac{V_g \text{ ml.}}{1} \times \frac{\text{Std. Temp. } ^\circ\text{K}}{\text{Bath Temp. } ^\circ\text{K}} \times \frac{\text{Pressure change}}{\text{Std. barometer}} \times \begin{matrix} \uparrow \\ A \end{matrix}$$

$$\frac{1 \text{ mole}}{22414 \text{ ml.}} \times \begin{matrix} \uparrow \\ B \end{matrix} \frac{253.82 \text{ g. I}_2}{1 \text{ mole}} \times \begin{matrix} \uparrow \\ C \end{matrix} \frac{1}{\text{Sample weight}} \times \begin{matrix} \uparrow \\ D \end{matrix}$$

$$\frac{100 \text{ g. sample} \times \text{HIV units}}{1 \text{ g. I}_2} = \text{HIV.} \quad \begin{matrix} \uparrow \\ E \end{matrix}$$

V_g is the volume of the flask and manometer minus the displacement of the solid and liquid material contained in the flask. The average temperature of the scale portion of the manometers is about 27°C . and a glass scale manometer at this temperature would require a reading of 763.54 mm. to read 760.0 when corrected for the temperature and glass scale. It is easier to use this figure in the equation than to correct each individual manometer reading. The pressure change is the $\Delta H'$ before hydrogenation minus the $\Delta H'$ after hydrogenation after this difference has been corrected for inaccuracies in the manometer scales, and is referred to as $\Delta H''$. Examination of three manometers from the set currently being used revealed inaccuracies in the scales. These are approximately the same on all manometers and can be fairly well corrected by reducing the difference between $\Delta H'$ before and $\Delta H'$ after by 0.3 mm. Thus the corrected $\Delta H''$ is

$$(\Delta H' \text{ before} - \Delta H' \text{ after}) - 0.3 \text{ mm.}$$

The equation to point A is the corrected

$$\frac{V_g \text{ ml}}{1} \times \frac{\text{std temp}}{\text{bath temp}} \times \frac{\Delta H' \text{ before hydrog.}}{\text{std barometer}} -$$

$$\frac{V_g \text{ ml}}{1} \times \frac{\text{std temp}}{\text{bath temp}} \times \frac{\Delta H' \text{ after hydrog.}}{\text{std barometer}}$$

which does not show the partial pressure due to solvent vapor pressure since the same figure would appear in both parts and

thus cancel out. (Actually this may be in error if the vapor pressure changes upon tipping of the sample but the thermometer is depended upon to compensate for this.) This much of the equation gives the uptake of hydrogen in ml. (s.t.p.).

The equation to point B is A divided by 22414 ml. per mole, giving moles of hydrogen uptake, while at point C we have B converted to the equivalent grams of iodine. By dividing C by the grams of sample, we have at D the grams of iodine per gram of sample. The equation to point E is D divided by $\frac{1 \text{ g I}_2}{100 \text{ g. sample}}$ per HIV unit, converting D to 100 gram basis, with the resultant answer being HIV.

Assuming that the manometer temperature does not vary enough from 27° to significantly affect the results (actually 1° C. changes the results 1 part in 6000), that portion of the equation except gas volume, pressure change and sample weight, is constant for a given bath temperature. For a bath temperature of 60° C., it has the numerical value of .001215970 and the dimensions of $\frac{\text{grams sample} \times \text{HIV units}}{\text{mm. Hg} \times \text{ml}}$ so that when multiplied by $\frac{(V_g \text{ ml})}{\text{grams sample}} \times \Delta H'' \text{ mm. Hg}$, all the dimensions cancel out except HIV units and we have our answer. The gas volume and the sample weight can be calculated even before actual hydrogenation and when entered into the equation, give a K_f for a particular flask-manometer-sample combination, which then needs only to be multiplied by the

corrected $\Delta H''$ to give HIV. This K_f should in the case of butterfat, have a value of 0.18 to 0.21 since if it is less, the final manometer reading may be off the scale, whereas if it is more, the determination is less sensitive.

In calculating the displacement of materials placed in the flasks, multiply the weight of glass beads and vials by 0.447 ml/gram. The weight of beads is assumed to be 2.150 g. The density of fat at 60° is about 0.8896 \pm 0.0010 g/ml., so the weight of fat is multiplied by 1.124. (In case a bath temperature other than 60° C. is used, the coefficient of expansion for fat is 78.34×10^{-5} ml/ml/° C., (172). The coefficient of expansion of propionic acid is 1.102×10^{-3} ml/ml/° C. Therefore, to find the displacement of the acid at bath temperature, multiply this figure by bath temperature minus pipetting temperature and the result by the volume pipetted. Add this figure to the original volume.

The figure of 253.82 g I₂ per mole is based on the 1951 International atomic weight of 126.91 rather than the previously accepted figure of 126.92. Whenever using this figure to calculate a HIV to be compared with the IV, be sure the IV is calculated using the same figure.

6. Hydrogenation procedure adopted

Since the listing of the various steps in the hydrogenation procedure are interspersed among the discussions of the development of the various steps, a description of the hydrogenation procedure as adopted is listed here. However, not included at this point are detailed descriptions of glassware cleaning, hydrogen generation and purification, catalyst preparation, and equation derivation.

a. Apparatus. The equipment needed for the hydrogenation includes:

Analytical balance (Ainsworth model DLB).

Small water bath for keeping samples melted.

Pipettes, one for each fat (six inch lengths of 6 mm. tubing drawn down to a tip).

Catalyst vials.

Sample vials.

Desiccator for holding catalyst and for sample and catalyst vials.

Hydrogenation flasks as described on page 97, with C-clamps or springs for securing lids.

Modified Warburg manometers as described on page 103.

One 5 ml. pipette.

Hydrogen generation apparatus as described on page 135.

Warburg shaker and bath with adequate temperature control.

Clock.

Portable hot plate (a small electric clothes pressing iron was used).

Forceps shaped for handling sample and catalyst vials.

Small spatula with tip (15 mm.) bent to form an angle of approximately 135° with axis of handle.

Glass beads, 6 mm. diameter.

b. Reagents and solvents. The necessary reagents and solvents include:

Hydrogen, generated and purified as described on page 135.

Propionic acid.

Catalyst prepared as described on page 141.

Joint sealer. A mixture of six parts (by weight) of silicone high vacuum grease and one part Celite.

c. Procedure. Into numbered catalyst vials weigh 150 mg. \pm 1 mg. catalyst and record weight of the vials (unless these show evidence of chipping, these need be weighed only about every third or fourth time of use). This weighing should be done well in advance of use and the vials of catalyst returned to the desiccator to insure that catalyst at

time of use will not have absorbed moisture. Accurately weigh samples into the numbered sample vials, the approximate size being determined by the equation

$$\text{Approx. wt. sample} = 250 \text{ mg.} \times \frac{\text{Approximate gas volume}}{\text{Anticipated Hydrogenation value}}$$

Place samples in a desiccator until ready to begin hydrogenation. In addition to the samples, place into at least one other sample vial, approximately the same amount of fat, for use in the thermobarometer. It is not necessary to weigh this. Use the same number of drops from the pipette as for one of the weighed samples.

On the ground joint of the body of the hydrogenation flasks and on the counterpart on the lids, place a thin film of the joint sealer. Place the sample vials in the sidearms of the flasks. By use of forceps and bent spatula, place catalyst vials into hydrogenation flasks, setting vials upright on bottom near sidearm. In each flask, place 10 glass beads. Measure 5 ml. propionic acid, pick up hydrogenation flask, tip so as to lay catalyst vial over at 45° angle and allow a small amount of solvent to flow slowly down the inside of the vial. As soon as all catalyst has been covered, set flask upright, being careful not to upset vial, and fill vial to within about 6-8 mm. of the top. Place the remainder of the 5 ml. propionic acid into flask. Note and record temperature of propionic acid. Place one or two drops of fat

into each flask (including thermobarometer, which should also contain propionic acid). Place lids on flasks and secure, rotating lid to insure a good seal. Where clamps are used, use a small square (10 mm.) cut from tygon tubing as a cushion.

Grease joints on manometer sidearms and connect flasks to manometers. Open stopcocks at top and bottom of manometers and adjust mercury columns to a height of about 200-230 mm. Unless present from previous run, add by means of long thin capillary, sufficient propionic acid to form a 20 mm. column on top of each mercury column. Close stopcock at bottom of each manometer and connect both arms of manometer to the evacuation and filling manifold of the hydrogen generation and purification apparatus. Using the portable hot plate underneath each flask sidearm, heat the fats just sufficiently to melt them.

By means of the vacuum pump, evacuate the flasks and manometers to a residual pressure of not more than 10 mm. Hg and maintain for 10 minutes. Be sure the liquid nitrogen traps on the apparatus are cold and properly functioning. After the ten minutes, slowly admit hydrogen to within 100 mm. of atmospheric pressure and then re-evacuate. As soon as the pressure has been reduced to 10 mm. or less, readmit hydrogen. Continue in this manner to a total of four evacuations. On the fourth filling with hydrogen, close off the thermobarometer

flask and manometer when the hydrogen pressure is 50-60 mm. less than atmospheric pressure and continue filling the others to about 15 mm. less than atmospheric pressure. Close the stopcocks at the tops of the manometers and disconnect the manifold.

Just before placing the flasks in the previously heated Warburg bath, shake the flasks gently to tip the catalyst vials.

After shaking for 45 minutes, allow the flasks to remain undisturbed for one minute. Lower the mercury columns below the reading mark, slowly raise to mark and read, estimating to 0.1 mm. Read temperature of bath. Repeat for two more readings of manometers and thermometer, followed by a reading of barometer.

Average the three readings of the manometer and record this average as ΔH ; unless the total range of the three exceeds 0.4 mm. If just two are in this range, average them, otherwise disregard all.

From the ΔH for each sample, subtract the ΔH for the thermobarometer and record the result (with proper sign) as $\Delta H'$. Thirty minutes after stopping for the first reading, again discontinue shaking and allow the flasks to remain undisturbed for one minute, followed by reading as before. Continue reading at 30 minute intervals until sufficiently constant $\Delta H'$ values are obtained. Generally three consecutive

readings covering a total range of 0.2-0.3 mm. can be obtained. When the pressure has become constant, remove each flask from the bath and tip the sample from the sidearm, washing out the sidearm with some of the solvent. Return the flasks to the bath and continue shaking. After about 30-45 minutes of shaking, continue manometer readings as before until a constant pressure is obtained.

d. Calculations. The derivation of the equation is discussed on page 147. Add the flask and manometer volumes and from the total, subtract the displacement of vials, beads, sample and solvent. Multiply this gas volume by the equation constant (which for a 60° reaction is 0.00121597) and divide by sample weight in grams. This gives a constant for the particular flask-manometer-sample combination. From the constant manometer reading before hydrogenation, subtract the constant reading after hydrogenation and from this subtract a scale correction of 0.3 mm. Multiply this corrected ΔH by the flask constant obtained as above and express the results as hydrogenation iodine value.

7. Hydrogenation of methyl esters

a. Esters used. In an attempt to check the accuracy of the newly developed hydrogenation method, the methyl esters of several unsaturated fatty acids were hydrogenated. The esters were obtained from the Hormel Institute, Austin, Minn.

There were two different lots of methyl oleate, two of methyl linoleate and one lot of methyl linolenate. The methyl oleate had been prepared by fractional distillation and repeated recrystallization from methyl esters of olive oil fatty acids. The methyl linoleate and methyl linolenate had been prepared by debromination of repeatedly recrystallized tetrabromostearic acid and hexabromostearic acid respectively. Lot 3 of methyl oleate and lot 1 of methyl linoleate had been held at -25° in the original sealed containers during four or five years after their receipt. The other three lots had been obtained four months prior to analysis and likewise had been held at -25° .

Table 9 gives the analysis as supplied for Hormel Institute for the lots of methyl oleate while Table 10 gives the analysis supplied with the lots of methyl linoleate and methyl linolenate. These analyses were made according to the method of Brice, Swain, Schaeffer and Ault (390). It may be noted that there were apparently considerable amounts of impurities in some of the lots, particularly in lot 12 of methyl oleate. However, it should be pointed out that the method of Brice, Swain, Schaeffer and Ault is a method for determining polyunsaturated compounds. As a commentary on a table included in their report they state (390, p. 224):

Calculated values for oleic and saturated acids are not included in the table. These acids, of course, can be calculated from the proportions of polyunsaturated

Table 9

Analyses Supplied with Methyl Oleate^{ab}

| | Lot 3 | | Lot 12 | |
|------------|---------------------|------------------------|---------------------|------------------------|
| | Conjugated acids | Nonconjugated acids | Conjugated acids | Nonconjugated acids |
| Saturated | xxx | none | xxx | 1.65 |
| Monoenoic | xxx | 99.79 | xxx | 98.28 |
| Dienoic | none | 0.23 | 0.02 | 0.08 |
| Trienoic | none | none | none | none |
| Tetraenoic | none | none | none | none |

^aData taken from mimeographed sheets supplied with product by Hormel Institute, Austin, Minnesota.

^bAnalysis according to Brice *et al.* (24), expressed as per cent methyl esters of C₁₈ fatty acids.

constituents indicated by the spectrophotometric data, their theoretical iodine numbers and the iodine number of the sample.

The calculation of oleic and saturated acids on this basis is therefore based on the assumption that the iodine value method (in this case the Wijs method) gives a true measure of unsaturation, an assumption which is open to some question.

Two further points should be mentioned with regard to the data on methyl linoleate and methyl linolenate. No mention is made in the report of analysis of non-conjugated polyunsaturated constituents nor of saturated and monoethenoid constituents.

Table 10

Analyses Supplied with
Methyl Linoleate and Methyl Linolenate^{ab}

| | | Lot 1 Linoleate | Lot 14 Linoleate | Lot 9 Linolenate |
|-------------|---------------|--------------------|---------------------|---------------------|
| Dienoic: | not more than | 0.17 | 0.15 | 0.60 |
| Trienoic: | not more than | 0.001 | 0.04 | nil |
| Tetraenoic: | not more than | trace | 0.003 | 0.008 |

^aData taken from mimeographed sheets supplied with product by Hormel Institute, Austin, Minnesota.

^bConjugated polyunsaturated constituents (from ultra-violet absorption data), expressed as percentage of methyl esters of C₁₈ fatty acids.

With regard to the conjugated polyunsaturated constituents, the phrase "not more than" precedes the figures given.

b. Weighing. Because of their high degree of unsaturation and therefore probable ease of oxidation, extreme precautions were taken in the weighing of the esters. Only red light was used for illumination and attempts were made to handle the material only in an inert atmosphere. Once the seal on the original tube of ester was broken, the tube was placed in a hydrogenation flask which had previously been flushed with inert gas. Then the lid was placed on the flask and a continuous stream of gas was supplied through the small opening in the lid. Sample vials placed in hydrogenation flasks were thoroughly flushed and after the flask, with lid

in place, had been flushed, a glass stopper was placed in the opening in the lid. After getting a tare weight on the flask and its contents, only the stopper was removed while the sample was introduced. The sample was handled with a specially shaped pipette drawn out to a long, very thin tip. The pipette tip was doubled back upon itself twice with two bends of 180° in an attempt to prevent drippage from the tip during handling. After filling, the tip was wiped with a dry cleansing tissue before being introduced into the tared flask. Care was taken to protect the pipettes from contamination by fat or esters after they had been heated for shaping. They were used for only one ester, not being cleaned and reused. Inert gas was drawn through each pipette just before the tip was immersed into the ester. After the desired amount of sample had been introduced, the flask was again flushed, stoppered and weighed.

The first attempts at weighing under the above conditions were made using carbon dioxide but the results were not satisfactory. Since the ground joints of the flasks were not lubricated, the diffusion of the heavy carbon dioxide may have been the cause of the slow but rather continuous decrease in weight. Nitrogen was next tried. At first, there were difficulties due to the cooling of the gas upon expansion as it left the cylinder. These were eliminated by passing the nitrogen through a coil immersed in a water bath about 5°

above room temperature.

c. Results. In addition to hydrogenation value, the iodine value according to the Rosenmund-Kuhnhehn method was determined on each ester. The results of these, together with Wijs iodine values as reported by Hormel Institute are shown in Tables 11 to 15. Also shown are theoretical values and the relationship of observed values to theoretical values.

With methyl oleate, the hydrogenation results on lot 3 (the older of the two lots) were below theoretical, both on the basis of the Hormel Institute analysis and on a pure compound basis. On lot 12 the results were above theoretical on both bases, but on both lots the results were nearer the theoretical values for a pure ester than for the values resulting from the analysis by Hormel Institute. In fact, with the fresher of the two lots, the deviation of the average hydrogenation iodine value was less than two parts per thousand from the theoretical value for the pure ester although individual values differed by as much as six parts per thousand from this value. The two individual values on lot 3 give almost perfect agreement, while the total range of the five values on lot 12 amounts to ten parts per thousand. The standard deviation is 0.351 hydrogenation iodine units. Since the analyses were apparently based upon a Wijs iodine value, it seems quite possible that the material had a higher degree of purity than was indicated, the low analyses being the

Table 11

Analytical Results on Lot 3, Methyl Oleate

| | | Values | Percentage of theoretical values based on | |
|---|------------------|--------------------|---|-----------------------|
| | | | Pure compound | Hormel Inst. analysis |
| Theoretical for pure ester | | 85.61 | 100.00 | 99.74 |
| Theoretical according to analysis by Hormel Institute | | 85.83 | 100.26 | 100.00 |
| Wijs iodine value | | 85.84 ^a | 100.27 | 100.01 |
| Rosenmund-Kuhnhehn iodine values | 5 min. reaction | 85.23 | 99.57 | 99.30 |
| | | 85.51 | 99.88 | 99.63 |
| | | 85.43 | 99.79 | 99.53 |
| | 20 min. reaction | | | |
| | 40 min. reaction | | | |
| | Overall average | 85.39 | 99.74 | 99.49 |
| Hydrogenation iodine values | | 85.11 | 99.42 | 99.16 |
| | | 85.14 | 99.45 | 99.20 |
| | Average | 85.13 | 99.44 | 99.18 |

^aData from mimeographed sheet supplied with product by the Hormel Institute, Austin, Minnesota.

Table 12

Analytical Results on Lot 12, Methyl Oleate

| | | Values | Percentage of theoretical values based on | |
|---|------------------|-------------------|---|-----------------------|
| | | | Pure compound | Hormel Inst. analysis |
| Theoretical for pure ester | | 85.61 | 100.00 | 101.54 |
| Theoretical according to analysis by Hormel Institute | | 84.31 | 98.48 | 100.00 |
| Wijs iodine value | | 84.3 ^a | 98.47 | 99.99 |
| Rosenmund-Kuhnhehn iodine values | 5 min. reaction | 84.34 | 98.52 | 100.04 |
| | | 84.77 | 99.02 | 100.55 |
| | 20 min. reaction | 85.05 | 99.35 | 100.88 |
| | | 85.47 | 99.84 | 101.38 |
| | 40 min. reaction | 84.82 | 99.08 | 100.60 |
| | | 84.77 | 99.02 | 100.55 |
| Overall average | | 84.87 | 99.14 | 100.66 |
| Hydrogenation iodine values | | 85.67 | 100.07 | 101.61 |
| | | 86.14 | 100.62 | 102.17 |
| | | 85.27 | 99.60 | 101.26 |
| | | 85.63 | 100.02 | 101.57 |
| | | 86.05 | 100.51 | 102.06 |
| Average | | 85.75 | 100.16 | 101.71 |

^aData from mimeographed sheet supplied with product by the Hormel Institute, Austin, Minnesota.

Table 13

Analytical Results on Lot 1, Methyl Linoleate

| | | Values | Percentage of theoretical values based on | |
|---|------------------|--------------------|---|-----------------------|
| | | | Pure compound | Hormel Inst. analysis |
| Theoretical for pure ester | | 172.39 | 100.00 | 100.00 |
| Theoretical according to analysis by Hormel Institute | | 172.39 | 100.00 | 100.00 |
| Wijs iodine value | | 172.4 ^a | 100.01 | 100.01 |
| Rosenmund-Kuhnenn iodine Values | 5 min. reaction | 158.54 | 91.97 | 91.97 |
| | | 158.61 | 92.01 | 92.01 |
| | | 158.86 | 92.15 | 92.15 |
| | 20 min. reaction | | | |
| | 40 min. reaction | | | |
| | Overall average | 158.67 | 92.04 | 92.04 |
| Hydrogenation iodine values | | 173.06 | 100.39 | 100.39 |
| | | 172.38 | 99.99 | 99.99 |
| | | 172.72 | 100.19 | 100.19 |
| Average | | 172.72 | 100.19 | 100.19 |

^aData from mimeographed sheet supplied with product by the Hormel Institute, Austin, Minnesota.

Table 14

Analytical Results on Lot 14, Methyl Linoleate

| | | Values | Percentage of theoretical values based on | |
|---|------------------|--------------------|---|-----------------------|
| | | | Pure compound | Hormel Inst. analysis |
| Theoretical for pure ester | | 172.39 | 100.00 | 99.98 |
| Theoretical according to analysis by Hormel Institute | | 172.43 | 100.02 | 100.00 |
| Wijs iodine value | | 171.7 ^a | 99.60 | 99.58 |
| Rosenmund-Kuhnhenh iodine values | 5 min. reaction | 152.96 | 88.73 | 88.71 |
| | | 154.17 | 89.43 | 89.41 |
| | 20 min. reaction | 156.59 | 90.83 | 90.81 |
| | 40 min. reaction | 156.69 | 90.89 | 90.87 |
| | | 156.56 | 90.82 | 90.80 |
| | Overall average | 155.39 | 90.14 | 90.12 |
| Hydrogenation iodine values | | 172.14 | 99.85 | 99.83 |
| | | 171.27 | 99.35 | 99.33 |
| | | 171.69 | 99.59 | 99.57 |
| | | 173.95 | 100.90 | 100.88 |
| | | 171.60 | 99.54 | 99.52 |
| Average | | 172.13 | 99.85 | 99.83 |

^aData from mimeographed sheet supplied with product by the Hormel Institute, Austin, Minnesota.

Table 15

Analytical Results on Lot 9, Methyl Linolenate

| | | Values | Percentage of theoretical values based on | |
|---|------------------|--------------------|---|-----------------------|
| | | | Pure compound | Hormel Inst. analysis |
| Theoretical for pure ester | | 260.37 | 100.00 | 100.20 |
| Theoretical according to analysis by Hormel Institute | | 259.85 | 99.80 | 100.00 |
| Wijs iodine value | | 259.0 ^a | 99.47 | 99.67 |
| Rosermund-Kuhnenn iodine values | 5 min. reaction | 231.63 | 88.96 | 89.14 |
| | | 228.16 | 87.63 | 87.80 |
| | 20 min. reaction | 232.62 | 89.34 | 89.52 |
| | | 232.17 | 89.17 | 89.35 |
| | 40 min. reaction | 229.40 | 88.11 | 88.28 |
| | | 230.91 | 88.69 | 88.83 |
| | Overall average | 230.82 | 88.65 | 88.83 |
| Hydrogenation iodine values | | 259.21 | 99.55 | 99.75 |
| Average | | 259.21 | 99.55 | 99.75 |

^aData from mimeographed sheet supplied with product by the Hormel Institute, Austin, Minnesota.

result of incomplete reaction with Wijs reagent.

While it is considered unlikely (in view of the conditions of storage), there is a slight possibility that the low results on the older lot may be the result of a slight oxidation. If hydroperoxides were present these might be expected to cause high results but with such prolonged storage, any hydroperoxides may have decomposed. If the hydroperoxides interacted with ethenoid linkages to form epoxy groups, while themselves being changed to hydroxy groups, this might lower the hydrogenation value.

The analyses submitted by the Hormel Institute on the lots of methyl linoleate give theoretical values in very good agreement with the theoretical values for pure esters, whereas the Wijs iodine value on lot 14 is appreciably below the theoretical value. On each of these lots the average of the hydrogenation iodine value is within two parts per thousand of the theoretical value. On lot 1 the standard deviation is 0.34 hydrogenation iodine units while the corresponding figure for lot 14 is 1.064.

The hydrogenation iodine value of the methyl linolenate, while below the theoretical figure, is above the Wijs iodine value. (Because of leakage in the equipment only one value was obtained on the methyl linolenate.)

The Rosenmund-Kuhnhehn iodine value on the methyl oleate, lot 3, was below the theoretical value and almost half a unit

below the Wijs value but was higher than the hydrogenation iodine value, which was a rather unusual occurrence in this study. There was very good agreement among the results of the three determinations, the standard deviation being 0.144 iodine units. Lot 12 showed an average Rosenmund-Kuhnhenh value which was 0.88 units below the hydrogenation value and was more than half a unit above the Wijs value. Hormel Institute had reported this lot to contain 1.65 per cent esters of saturated fatty acids though the hydrogenation value would tend to cast some doubt on this. Although the Rosenmund-Kuhnhenh values with a 20 minute reaction time were higher than those with either 5 or 40 minutes and the 5 minute values were lowest, the standard deviation of all values without regard to reaction time was only 0.373 iodine units on lot 12.

The polyunsaturated esters showed rather unusual results in the Rosenmund-Kuhnhenh determination. The results were approximately eight and ten per cent below theoretical results. Lot 1, the older lot, of methyl linoleate which had shown good agreement as regards hydrogenation, likewise showed good agreement among replicate iodine values, the standard deviation being 0.168 units. The average value, however, was eight per cent below the theoretical figure and slightly over eight per cent below the hydrogenation value. Lot 14 showed less agreement here as it had also shown with the hydrogenation. The standard error was 1.993 iodine units.

The results here were almost 10 per cent below both theoretical and hydrogenation values.

The methyl linolenate values showed an appreciable variation, the standard deviation being 1.722 iodine units. Here again the values at 20 minutes were the highest. The overall average was more than 11 per cent below the theoretical figures and was 10.95 per cent below the hydrogenation value.

If one assumes that the hydrogenation value is a correct measure of the unsaturation, an average of 32.85 double bonds per 100 molecules of methyl linolenate used does not react with Rosenmund-Kuhnnehn reagent while in lot 1 of the methyl linoleate (the lot giving the better agreement of the two linoleates) an average of 16.27 double bonds per 100 molecules do not react. This raises a question (as yet unanswered) as to whether there is a direct relationship here or perhaps only coincidence. The other lots of methyl linoleate showed on this same basis 19.46 double bonds not reacting per 100 molecules.

8. Hydrogenation of fats direct from -25° storage

During the development of the hydrogenation method, various butterfats were hydrogenated but, generally speaking, the pressures did not become constant either before or after hydrogenation. The values were, however, consistently higher than the Rosenmund-Kuhnnehn iodine values on the same fat.

Since equilibrium was not attained, it was not possible to get exact values. The differences between iodine and hydrogenation values obtained during this phase of the work were generally ca. 1.4 to 2.0 units.

In April 1950, the results shown in Table 16 were obtained for eight simultaneous hydrogenations of the same fat. While these results were in rather close agreement, results such as these could not always be obtained at that time. After two more years in the development phase much better agreement could be obtained as will subsequently be shown. The results do, however, show a very distinct variation from the iodine value of 32.0 on this fat.

If one takes for each sample, the average of the values at different times, excluding those footnoted (when the hydrogenation was obviously not complete) he finds that the average of the values for the different samples is 33.41 with a standard deviation of 0.136 iodine units. The values at different reading times on any one sample cover a range of 0.05-0.52 hydrogenation iodine unit with all but one being 0.15 unit or more.

If one were to compare the first readings (excluding those which were obviously not complete), the standard deviation would be about the same as above but the average value would be about 0.095 unit lower. Later work after the developmental phase of the hydrogenation was completed showed that

Table 16

Relationship of Hydrogenation Values to Time
After Start of Hydrogenation^a

| Sample | Minutes after start of hydrogenation | | | | | Average |
|--------|--------------------------------------|--------------------|-------|-------|-------|---------|
| | 45 | 75 | 120 | 205 | 265 | |
| 04 | 31.59 ^b | 32.61 ^b | 33.24 | 33.39 | 33.33 | 33.32 |
| 6 | 33.55 | 33.61 | 33.68 | 33.55 | 33.50 | 33.58 |
| 5 | 33.39 | 33.50 | 33.70 | 33.73 | 33.51 | 33.57 |
| 3 | 33.25 | 33.30 | 33.29 | 33.29 | 33.21 | 33.27 |
| 7 | 33.44 | 33.51 | 33.50 | 33.53 | 33.75 | 33.55 |
| 9 | 29.43 ^b | 31.39 ^b | 33.18 | 33.34 | 33.70 | 33.41 |
| 13 | 33.33 | 33.40 | 33.36 | 33.35 | 33.27 | 33.34 |
| 15 | 33.15 | 33.23 | 33.32 | 33.32 | 33.30 | 33.26 |

^aHydrogenation method used here not that finally adopted.

^bAverages figured without use of values marked with a superscript.

hydrogenation was usually complete in 45 minutes. However, here the values at two hours after start of hydrogenation show a standard deviation of 0.197 hydrogenation iodine units, although the average is the same as that found by averaging all readings.

In August 1950 a large number of determinations were made on a sample of milk fat obtained from Louisiana in April. Disregarding those determinations which were obviously slow in taking up hydrogen (whether because of inactive catalyst or

Table 17

Values on Replicate Hydrogenations of April
Louisiana Milk Fat^a

| August 1 | August 11 | August 16 | August 17 |
|----------|-----------|-----------|-----------|
| 37.55 | 37.75 | 37.26 | 38.02 |
| 37.51 | 37.57 | 37.49 | 37.74 |
| 37.52 | 37.53 | 37.42 | 37.66 |
| 37.30 | 38.18 | 37.28 | |
| 37.26 | 37.57 | 37.46 | |
| 37.53 | 37.63 | 37.68 | |
| | 37.51 | 37.77 | |
| | 37.22 | 37.65 | |
| | 37.66 | 36.72 | |
| | | 37.77 | |

^aHydrogenation method used here not that finally adopted.

for other reason) and taking the first reading after start of hydrogenation (which on this group of determinations were in almost every case at least one hour after start of hydrogenation and which seemed here to give as good agreement as any other method of figuring) the values shown in Table 17 were obtained. The iodine number of this fat was 35.76. These values range from 36.72 to 38.18 and have a standard deviation of 0.222 hydrogenation iodine units. At the time these determinations were made it was still not possible to obtain the desired equilibrium.

After the adoption of an all glass system with the use of a propionic acid layer above the mercury in the manometers, much better equilibration was obtained and the agreement among

replicate determinations was considerably improved. Table 18 gives the values obtained on a sample of March fat held at -25° , while Table 19 presents similar figures on a June fat.

For the March fat the range of values was 0.09 unit, with a standard deviation of 0.028 hydrogenation iodine unit. The Rosenmund-Kuhnhehn iodine value was 32.91. The difference between the iodine and hydrogenation values was 0.34 unit.

The June fat, as would be expected, showed considerably higher values. Furthermore, there was not as good agreement, the range of values being 0.23 unit and the standard deviation, 0.083 unit. There was a much greater difference between the hydrogenation iodine value and the iodine value here than with the March fat. The iodine value was 39.38, or 1.30 units less than the average of 40.68 for hydrogenation. Whether the greater difference between methods with the summer fat and the lower iodine values of polyunsaturated methyl esters result from the same cause is as yet undetermined.

Assuming an average glyceride molecular weight of 750 for milk fat, 100 grams of fat (the weight on which the iodine value is based) would contain 0.4 moles of fatty acid residue. One gram of iodine (or the halogen equivalent thereto) would be absorbed by 0.00394 moles of ethenoid linkage. Therefore, to change the iodine value one unit, 0.00394 moles of double bond must have failed to react. On the basis of the results obtained with the methyl esters tested it seems that

Table 18

Values on Replicate Hydrogenations of March Milk Fat^{ab}

| June 26 | June 30 | July 2 |
|---------|---------|--------|
| 33.25 | 33.25 | 33.29 |
| 33.23 | 33.24 | 33.27 |
| 33.27 | 33.20 | |

^aHydrogenation method used here was that finally adopted.^bRosenmund-Kuhnhehn iodine value was 32.91.

Table 19

Values on Replicate Hydrogenations of June Milk Fat^{ab}

| June 26 | June 30 | July 2 |
|---------|---------|--------|
| 40.66 | 40.78 | 40.71 |
| 40.67 | 40.62 | 40.82 |
| 40.76 | . | 40.59 |

^aHydrogenation method used here was that finally adopted.^bRosenmund-Kuhnhehn iodine value was 39.38.

approximately 10 per cent of the double bonds in those polyunsaturated esters failed to react. Cow milk fat is normally considered to be practically devoid of trienoic acids. If, then, the difference between the iodine and hydrogenation iodine values is due to a failure of 10 per cent of the linoleate to react, there must be present for each one unit difference 0.0394 moles of double bond in the form of linoleate or 0.0197 moles of linoleate. This figure, on the basis of an assumed glyceride molecular weight of 750 then represents 4.925 molar per cent of the total component acids in the fat. To obtain a difference of 1.30 units would necessitate 6.40 molar per cent linoleate. A larger glyceride molecular weight (other assumptions remaining unchanged) would require even more linoleate. Hilditch (96) cites a number of analyses of the component acids of milk fat and in the only case in which the figure for octadecadienoic acid exceeds 4.5 molar per cent, it is 5.0. The reason for the difference between the iodine and hydrogenation values is, therefore, still unexplained.

B. Study of Milk Fats During Oxidation

1. Objectives

a. Relationship of results of various analyses. Several methods commonly used to follow deterioration of fats and oils

were to be employed in an endeavor to find methods which would show a high degree of correlation with each other and with the amount of deterioration indicated by organoleptic observations during the useful life of the fat. It was considered that if such correlations could be found, they might give further insight into the mechanism of the process of autoxidation of fats.

b. Prediction of shelf life. It was hoped that a thorough examination of the results obtained might prove of value in indicating that one or more of the methods studied could be employed for predicting the useful shelf life of dry milk fat.

c. Effect of temperature. Fat oxidations are known to proceed at a more rapid rate with increase in temperature. Within the temperature range -20° F. to 100° F. the oxidative mechanism may or may not be the same. It was hoped that this study might throw some light on this aspect of milk fat oxidation.

d. Effect of season. Dairy products generally are considered to be more susceptible to the development of oxidized flavor if they are produced during winter than during summer. It is not definite whether the greater susceptibility during winter is a result of triglyceride oxidation or of other entities of the lipid fraction (cf., phospholipids in milk). It was hoped that this phase of the study would yield

preliminary data indicating whether or not the rate and mechanism of oxidation of dry milk fat are primarily functions of the glyceride structure as it is affected by season, or to variations among certain components (e.g., tocopherols) of the nonsaponifiable fraction of the dry milk fat.

2. Outline of experiment

a. Selection of samples. Because of the difference in the structure of the component fatty acids and also because of the difference in the content of tocopherol, the naturally occurring fat-soluble antioxidant of milk, a late winter (March) fat and an early summer (late June) fat were selected for study.

b. Storage. The fats selected were subdivided into aliquots of about 50 ml. each and stored at -25° , 4° , 25° and 40° .

c. Analyses made. The observations to be made on the samples were those for tocopherols, peroxides, and carbonyl compounds, iodine value, hydrogenation iodine value and organoleptic flavor score. In the beginning, these analyses were to be made each 18 days on samples stored at -25° and each 12 days on those at 4° . Samples stored at 25° were to be analysed every sixth day while those at 40° every second day. Provision was made in scheduling the analyses so that as the samples began to deteriorate rapidly as indicated by the rate

of change in the values, the intervals between analyses could be cut in half.

d. Study of results. The results of the various analyses were to be plotted against each of the other analyses. In making up these plots, the order of dependency of the factors was considered to be in the following decreasing order:

Time
Organoleptic score
Iodine value
Tocopherol content
Peroxide content
Carbonyl content
Hydrogenation iodine value.

Time was placed first because all other values would vary with it. The hydrogenation iodine value was placed last because the method was newly developed and it was desired to determine its reliability as a method of studying fats during oxidation. Because it was considered the best available criterion of fat deterioration, organoleptic score was placed second. Iodine value was next in order because it is a fairly accurate determination and is directly related to fat structure. Tocopherol content should be a function of the oxidative condition of the fat. Of peroxide and carbonyl values, the peroxide determination is believed more accurate.

Correlation coefficients were to be calculated between pairs of values. Since the course of the oxidative mechanism may change as the reaction proceeds, due to autocatalytic effects or to accumulation of oxidation products, the fats

stored at different temperatures, as well as those produced at different seasons were to be compared to different levels of organoleptic deterioration.

3. Methods

a. Preparation of samples. Cream was obtained directly from the separator from the market milk department of the college creamery, was chilled at 40° F. overnight and was churned in one-quart Mason jars with glass lids and rubber gaskets in a shaker with reciprocal action. The long axis of the jar was laid in the direction of the agitation. The butter was washed with cold water and packed in 4 oz. sample jars. The butter was melted in a water bath protected from light at temperatures not exceeding 50°. The sample jars were centrifuged for 15-20 minutes at 1500 r.p.m. in a No. 2 International centrifuge. The fat was filtered through a Whatman No. 12 filter paper in a heated funnel. The total fat from cream of a given season was combined, mixed thoroughly and placed in 2 oz. opal glass ointment jars. The caps were lined with aluminum foil.

b. Storage of samples. The requisite number of 2 oz. jars of fat for the storage period anticipated were stored at each of the four temperatures. The samples at -25° were stored in a commercial frozen-food cabinet (large home size), those at 4° in a similar cold-hold cabinet designed for higher

temperatures. The samples at 25° and 40° were stored in home made air baths; bath temperatures were constant to $\pm 0.2^\circ$. External Fenwall thermostats sensitive to changes in room temperature and circulating water temperature aided in maintaining a constant temperature.

All samples were first sharp frozen and were tempered to the particular temperature at which they were to be stored by holding in a water bath before placing in the storage units.

c. Hydrogenation. The apparatus, reagents, procedure and calculations for the hydrogenation method have been listed previously (page 151).

d. Iodine value. The iodine value determinations were made by the method described by Breazeale (22) using the Rosenmund-Kuhnhehn (185) reagent with a five minute reaction period. Approximately 25 ml. of the pyridine sulfate dibromide reagent was used for each sample; the thiosulfate was ca 0.1 N.

The procedure for milk fat is as follows: When the sample is removed from storage, it is placed in a small water bath to melt it if necessary and to keep it melted. Samples are weighed into small sample cups, using a sample of such size as to give a 100 per cent excess of brominating agent. Sample cup with sample is placed in iodine number flasks, care being used to prevent spillage of sample on side of flask. Ten ml. of CHCl_3 are added to each flask containing a sample

and to at least two more for use as blanks. The flasks are then swirled to dissolve the fat and placed in a 25° air bath. A sample is removed from the box and about 25 ml. of approximately 0.1 N pyridine sulfate dibromide are added. The exact amount of brominating agent need not be known so long as it is the same for blanks and all samples. The time is noted and two drops of syrupy phosphoric acid are placed in the trough of the flask which is then given one swirl and placed back in the constant temperature box. Exactly five minutes from the addition of the brominating agent, the flask is removed from the box and 6.5 ml. 10 per cent KI are added, approximately the first milliliter being placed in the trough before careful removal of the stopper. After mixing the KI with the residual pyridine sulfate dibromide in the flask, $\text{Na}_2\text{S}_2\text{O}_3$ (about 0.1 N but normality known exactly) is added from the burette until a very light straw color remains. Add 2 ml. 1 per cent starch solution and 50 ml. H_2O , rinsing down the trough and stopper with the water. Continue titration to the disappearance of the blue color of the starch-iodine complex. Shake vigorously and if blue color returns, dispel it with more thiosulfate. Record the amount of thiosulfate used. Blanks are treated in the same manner as the determinations.

e. Determination of peroxide content. The method used for the determination of peroxides was essentially that of Hills and Thiel (98) as modified by Handwerk (87). The

reaction temperature used was 50° as recommended by Hills and Thiel rather than 60° as used by Handwerk. In this study, mixing was accomplished by inverting the cylinders and returning to an upright position four times after each addition of reagent, each time allowing the air bubble to move completely from one end to the other. Handwerk had specified shaking as the method of mixing.

Cuvettes were matched at 510 m μ in the manner described by Van Devender (212b), using cobaltous chloride (5.95 g. plus 2 ml. conc. HCl, made to 100 ml. with distilled water).

The reference curve was prepared in the manner described by Handwerk (87) except that the volume was made to only 9.90 ml. with benzene-methanol (70:30) and one drop of HCl solution (ca. 0.25 N) was added after the addition of one drop of NH₄SCN solution. The preparation of the standard solutions employed is presented in Table 20.

The procedure with milk fat is as follows: Accurately weigh about 1 g. fat into a 50 ml. glass-stoppered amber volumetric flask. Dilute to 50 ml. with benzene-methanol (70:30 by vol.). From this, pipette 1, 3, 6 or 9 ml. of fat solution into 10 ml. g. s. graduated actinic mixing cylinder, the amount being such as to give at time of reading a transmittance preferably in the range of 80-95 per cent. If the peroxide range of sample is unknown, use three or four of the above amounts. Add benzene-methanol to a total of 9.9 ml.

Table 20

Amounts of Solutions
For Preparation of Peroxide Reference Curves

| Solution no. | ml. Solution | Approximate meq. iron per cylinder ^a |
|----------------|--------------|---|
| 2 ^b | 1 | .0028 |
| 2 | 2 | .006 |
| 2 | 5 | .014 |
| 2 | 8 | .023 |
| 3 ^c | 2 | .028 |
| 3 | 4 | .056 |
| 3 | 7 | .099 |
| 3 | 9 | .129 |
| 1 ^d | 2 | .143 |
| 1 | 3 | .220 |
| 1 | 4 | .280 |
| 1 | 5 | .350 |

^aBased on 7.162 meq. per ml. of stock solution.

^bTwo ml. solution 1 diluted to 50 ml.

^cTen ml. solution 1 diluted to 50 ml.

^dOne ml. stock solution diluted to 100 ml.

Add 1 drop NH_4SCN solution (30 g./100 ml. dist. H_2O), mix by inverting four times, add 1 drop of FeCl_2^a solution and again mix with four inversions. Heat for exactly three minutes at 50° in water bath. Cool for five minutes in water bath at room temperature. Pour into cuvettes and read against blank containing benzene-methanol and set for 100 per cent transmittance at 510 millimicrons.

Prepare a reagent blank in the same manner as for a sample except that benzene-methanol solvent is employed instead of fat solution. Prepare fat blanks with the same amount of fat as is used in determinations and in same manner except that one drop distilled water is used in the place of one drop FeCl_2 solution. Complete these blanks and read them in the same manner as for samples.

The standard regression determined (193) between log. percentage transmittance (x) and $\mu\text{eq. peroxide}$ in the aliquot read (y) is:

$$y = 0.55525 (1.9987 - x).$$

To calculate the peroxide content of the fat, subtract from the indicated peroxide content (according to the equation calculated above) of the sample the sum of the indicated peroxide content of the fat blank and the reagent blank. Then

^aDissolve 0.4 g. $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ in 50 ml. H_2O , add slowly a solution of 0.5 g. $\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$ in 50 ml. dist. H_2O . Centrifuge 5 min. at 1000 r.p.m. Decant supernatant into a brown bottle and flush with nitrogen.

correct for dilution and convert to a one g. basis. If P/g fat is the peroxide content of the fat and P_S , P_F , and P_R are the indicated peroxide content of the sample, the fat blank and the reagent blank respectively, then the equation becomes:

$$\frac{P}{g. \text{ fat}} = P_S - (P_F + P_R) \times \frac{50}{\text{ml. fat sol. used}} \times \frac{1}{g. \text{ fat in 50 ml. sol.}}$$

The answer is in μ eq. peroxide per g. of fat, which is the same as meq. peroxide per kg. of fat.

f. Determination of carbonyl compounds

(1) Method. The method used in this study for the determination of carbonyl compounds was that of Lea (122) except that the reagent was prepared from crystalline sodium bisulfite and the bottles containing the fat solution and bisulfite solution were flushed with nitrogen prior to shaking.

The following procedure was used on milk fat: Weigh one g. samples of fat into 70 ml. glass-stoppered bottles. To each of these bottles plus two bottles as blanks, add 2 ml. benzene (to dissolve fat) and 20 ml. bisulfite solution. Flush each bottle with nitrogen for about 30 seconds. Place bottles horizontally in shaker (Fisher-Kahn type) with stoppers held securely in place and with axis of bottles in the direction of shaking. Cover to protect from light and

shake one hour. Transfer contents of bottles to 50 ml. centrifuge tubes having a conical bottom. Centrifuge (Adams angle head clinical centrifuge) five minutes at 1000 r.p.m. Pipette 15 ml. aqueous solution into a 125 ml. Erlenmeyer flask and add 2 ml. 1 per cent starch solution. Add 1 N iodine until blue color persists for one or two seconds. Then add 0.05 N iodine until color remains for one or two seconds. After this, add 0.002 N iodine, adding one drop more than enough to give a color remaining one or two seconds. Note carefully the color. Add 3 g. NaHCO_3 , mix and titrate quickly with 0.002 N iodine to same color. Treat the two blanks similarly.

While the aqueous phase in the bottle amounted to 20 ml., only 15 ml. of this was titrated. The equation for calculating the carbonyl content is:

$$\frac{4}{3} \times \frac{(\text{ml. for sample} - \text{ml. for blank})}{\text{sample weight in g.}} \times \frac{\text{normality}}{2000}$$

= mmol. of carbonyl per kg. of fat.

g. Tocopherol determination.

(1) Method. Tocopherols were determined in this study by the method of Bird et al. (13b) using Floridin (treated with HCl and SnCl_2) for the removal of interferents after saponification of the fat. The tocopherol content was calculated on the basis of the color developed by the reaction

Table 21

Amounts of Solutions
For Preparation of Tocopherol Reference Curves

| Solution no. | ml. Solution | mcg. Tocopherol per cylinder ^a |
|----------------|--------------|---|
| 2 ^b | 0.5 | 5 |
| 2 | 2.0 | 20 |
| 2 | 4.0 | 40 |
| 2 | 6.0 | 60 |
| 2 | 8.0 | 80 |
| 1 ^c | 1.0 | 100 |
| 1 | 1.5 | 150 |
| 1 | 2.0 | 200 |
| 1 | 2.5 | 250 |
| 1 | 3.0 | 300 |
| 1 | 3.5 | 350 |

^aBased on 20 mg. tocopherol per ml. original solution.

^bFive ml. original solution diluted to 100 ml.

^cFifty ml. original solution diluted to 100 ml.

between ferrous iron and alpha, alpha'-dipyridyl. The ferrous iron results from reduction of ferric iron by the tocopherols. The reference solutions were prepared as indicated in Table 21. Reagents were prepared as indicated by Bird et al. (13b). Cuvettes were matched by the method of Van Devender (212b) using cobaltous chloride (5.95 g. plus 2 ml. HCl made to 100 ml. with distilled water) at 520 m μ .

For the determination on milk fat, proceed as follows. Weigh 10 g. fat into a 150 ml. standard taper amber extraction flask, add 5 ml. of 5 per cent pyrogallol and mix thoroughly

by rotary motion. Add 20 ml. 3.5 N methanolic KOH and mix. Place flask on lower end of standard taper water jacketed condenser and heat at 80° (water bath) for 10 minutes after the solvent begins to drip from the condenser tip. Rinse condenser (into flask) with 5 ml. methanol and remove flask from condenser. Add 40 ml. distilled water, mix, add 15 ml. methanol, mix and cork flask. Cool to room temperature in a room temperature water bath.

Transfer the cooled saponification mixture to a 250 ml. glass-stoppered, amber, Squibb type separatory funnel, rinsing the extraction flask into the funnel with small portions of distilled water (total 60 ml.) and small portions of peroxide free ether (total 80 ml.).

Shake funnel and contents vigorously ten times and after phases have separated run aqueous layer into clean 250 ml. beaker. Transfer the ether solution (through mouth of funnel) to a 250 ml. amber Erlenmeyer flask, rinse the separatory funnel with three 10 ml. portions of ether and rinse the mouth of the funnel after each transfer. Cork this flask and hold.

Return soap solution to the funnel and extract twice as above with 50 ml. portions ether, rinsing beaker into funnel with ether used in extraction. Transfer the second ether extract to flask containing the first extract. To third extract in funnel, add the first and second extracts. Wash

the combined extracts successively with 50 ml. 1 per cent aqueous KOH, 50 ml. 2 per cent Calgon, and two 50 ml. portions of distilled water. After the extracts have been shaken with KOH, add 20 ml. saturated NaCl and invert the funnel twice to prevent the formation of a three-phase system.

Transfer washed ether solution to a 300 ml. amber Erlenmeyer flask, add 10-15 g. anhydrous sodium sulfate and dry for at least two hours. Transfer dried ether solution to a 250 ml. standard taper amber extraction flask, rinsing the sodium sulfate and Erlenmeyer flask three times with 10 ml. ether. Evaporate solvent at 40-50° under vacuum, using a water bath for heating. When the ether is evaporated, submerge the flask to the bottom of the ground neck in a 70° bath for 30 seconds, and cool. Add 2 ml. benzene in the small reservoir at the top of the evaporation apparatus, close the stopcock between the apparatus and the aspirator and allow the benzene to flow slowly into the extraction flask, breaking the vacuum after the benzene is added. Transfer the residue with successive small (3-4 ml.) portions of benzene to a 25 ml. glass-stoppered amber volumetric flask and make to volume with benzene.

Pipette 10 ml. of this benzene solution onto the freshly prepared Floridin column^a, using care not to disturb the top

^aPrepare Floridin adsorption columns according to the method of Bird et al. (13b) as follows: Seal together a 200 mm. length of 9 mm. pyrex tubing and a 60 mm. length of 18 mm.

of the column. Wash the column five times with 5 ml. benzene each time, in each case adding the aliquot of benzene just as the upper surface of the previous aliquot reaches the surface of the Floridin; allow solution and washings to pass through the column at atmospheric pressure. Collect sample and washings in a 150 ml. standard taper amber extraction flask.

Using the evaporation apparatus, evaporate the benzene under vacuum at 45° . Keep the water bath surface below the surface of the liquid in the flask at all times. When dry, cool to room temperature, close the stopcock to the aspirator and add 2 ml. benzene through the reservoir at the top of the evaporation apparatus. Transfer the residue to a 25 ml. glass-stoppered amber mixing cylinder with small (2 ml.)

tubing. To the other end of the 9 mm. tubing seal a 60 mm. length of 3 mm. (i.d.) tubing. Grind the open end of this small tubing at an angle as on a funnel tip. After cleaning in chromic acid, pack the upper end of the small tube with glass wool. Weigh 1.5 to 2.0 g. Floridin XXS (Floridin Co., Warren, Pa.) into a 50 ml. beaker and add about 0.25 g. SnCl_2 and 5 ml. c.p. conc. HCl . Heat to boiling and pour into the adsorbent receptacle tube. Rinse with two 5 ml. portions absolute ethanol and five 5 ml. portions benzene. In each case, add a portion of liquid just as the upper surface of the preceeding portion reaches the surface of the adsorbent. Do not permit the adsorbent surface to become dry or exposed to air. While the last portion of benzene is on the adsorbent, cork the upper end of the tube and remove suction.

portions of benzene, bringing volume to exactly 10 ml.

Add 2 ml. color development reagent and make to 25 ml. with absolute ethanol. Stopper, mix, pour into cuvette and read against a blank made with 10 ml. purified benzene, 2 ml. color development reagent and 13 ml. ethanol. Read at 520 $m\mu$ exactly ten minutes after addition of color development reagent.

Using the values obtained with the alpha-tocopherol standard, plot a regression equation (193) letting x = logarithm of per cent transmission and y = mcg. tocopherol (per 25 ml. colored solution).

Correct the readings on milk fat for cuvette differences and, by means of the equation (or a table constructed using it), convert the readings to mcg. of tocopherol per 25 ml. colored solution. The 10 ml. solution used in color development represents 4 g. fat. The equation therefore becomes

$$\text{mcg. tocopherol/g. fat} = 0.25(\text{mcg. tocopherol/25 ml. solution})$$

The 10 minute reading is assumed to yield the total tocopherol content of milk fat.

h. Organoleptic observations. Several persons tasted the fat and each individually decided upon the flavor present and selected a numerical rating for the fat from the following score card:

| | |
|----------------------------------|-----|
| Fresh | 10 |
| Lacking fresh flavor | 9 |
| Stale | 8 |
| Oily | 7 |
| Slightly oxidized | 6 |
| Oxidized | (5 |
| | (4 |
| Badly oxidized | 3 |
| Metallic | 2 |
| Tallowy or fishy | 1 |
| Bleached and tallowy or fishy | 0 |

If differences occurred among the judges they attempted to agree on a flavor criticism and a numerical rating as a result of consultation among them.

4. Results

a. General statements. Correlation coefficients were calculated for the entire time of storage for all combinations among the several analyses. In addition, they were calculated for some series of fats to various levels of oxidation. For example, although the June fats at all temperatures except -25° were compared to a degree of deterioration indicated by flavor scores of 6 and 3, in no case did the March fat reach a score of 3, while with the June fat, it was impractical to use

comparisons at a score of 7 as was done with the March fat.

Since the number of determinations and consequently the number of degrees of freedom varied considerably with the different series, the mere citing of the value of "r", the correlation coefficient, would be of little significance unless one were also to state the degrees of freedom. Therefore, for the sake of uniformity and as a convention of terms, in discussing the results, the terms "highly significant" or "very significant" will be used whenever there is a probability of 0.01 (or less) of obtaining such a correlation coefficient by chance alone. Likewise the terms "significant" and "possibly significant" will be used for probabilities of less than 0.05 and 0.10 respectively. In general, a statement of "not significant" can be taken to mean a probability in excess of 0.10. It must be borne in mind that with a large number of calculations such as is involved here, it is almost inevitable that by chance there will be some very high values of "r" where there is no genuine significance. Nevertheless, for the sake of convention in discussing the results, the terms mentioned will be used throughout the thesis but where it appears from the data on members of the same series that there is no true significance, an endeavor will be made to point out this fact.

The coefficient of regression has been calculated for the full duration of storage for all combinations of analyses.

These are shown in Table 31. In some cases, whereas the individual members of a series do not show significance as determined by the correlation coefficient, the fact that all (or almost all) of the coefficients of regression in a series have similar sign may be indicative of a trend and so will be mentioned in some cases.

b. Changes with passage of time.

(1) Hydrogenation iodine values. The correlation coefficients of the relationships between the hydrogenation iodine values and the number of days in storage were not significant for either the March or the June fat at any temperature to any degree of oxidation that occurred. However, in all eight cases the coefficient of regression was found to be positive, indicating a slight tendency for the hydrogenation value to increase with time, the highest temperature having a greater influence on the rate of increase than the other temperatures involving the same fat. The trend is not great enough nor definite enough to enable one to say with certainty whether it is related to the increase in peroxide content in a causal relationship, is mere chance, or results from the fact that both the hydrogenation value and the peroxide content are related to some other factor.

(2) Iodine values. The correlations of the iodine values with time are of doubtful significance, though there does seem to be a tendency toward significance. The March

sample stored at 40° exhibits a tendency for the iodine value to increase with time, though for the full storage time of 19 days, it is not a significant tendency. Increases to 9 days (flavor score 7) and to 13 days (flavor score 6) are significant. Therefore it is possible that the value decreases during the latter part of the storage period. On the other hand, all other samples are inclined to show a decrease in iodine value with the passage of time, and with the June sample at 40° calculations seem to indicate significance, while with both fats at 25° the results are possibly significant.

(3) Peroxide content. Both the March fat and the June fat at 25° and at 40° show highly significant correlations between the peroxide content and time in storage. (Figures 9 and 10) At 25° the regression coefficients are approximately equal (+ 0.026 and + 0.029) while at 40° that for the March fat is almost 50 per cent larger than that for the June fat (+ 0.156 and + 0.107 meq. per kg. of fat per day respectively). This would be in agreement with the concept commonly held in the dairy industry that a winter fat is more easily oxidized than is a summer fat. In connection with these results it might be pointed out that while the results of the correlation of hydrogenation value with time were not significant, the regression coefficients for those values indicated a greater tendency for the hydrogenation value of the March fat to increase with time than was the case with the June fat. If

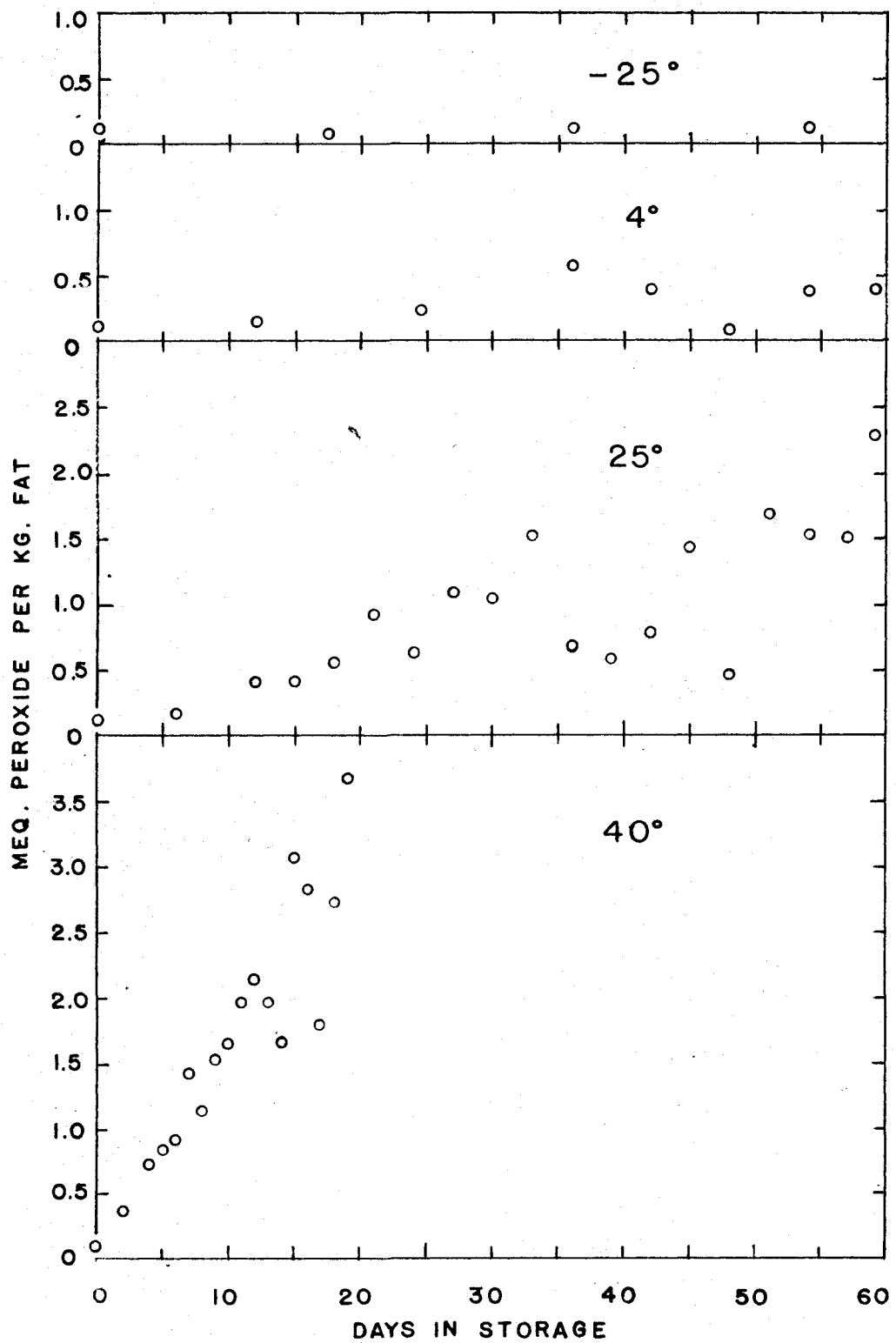


Figure 9. Changes in peroxide content of March fat during storage at various temperatures.

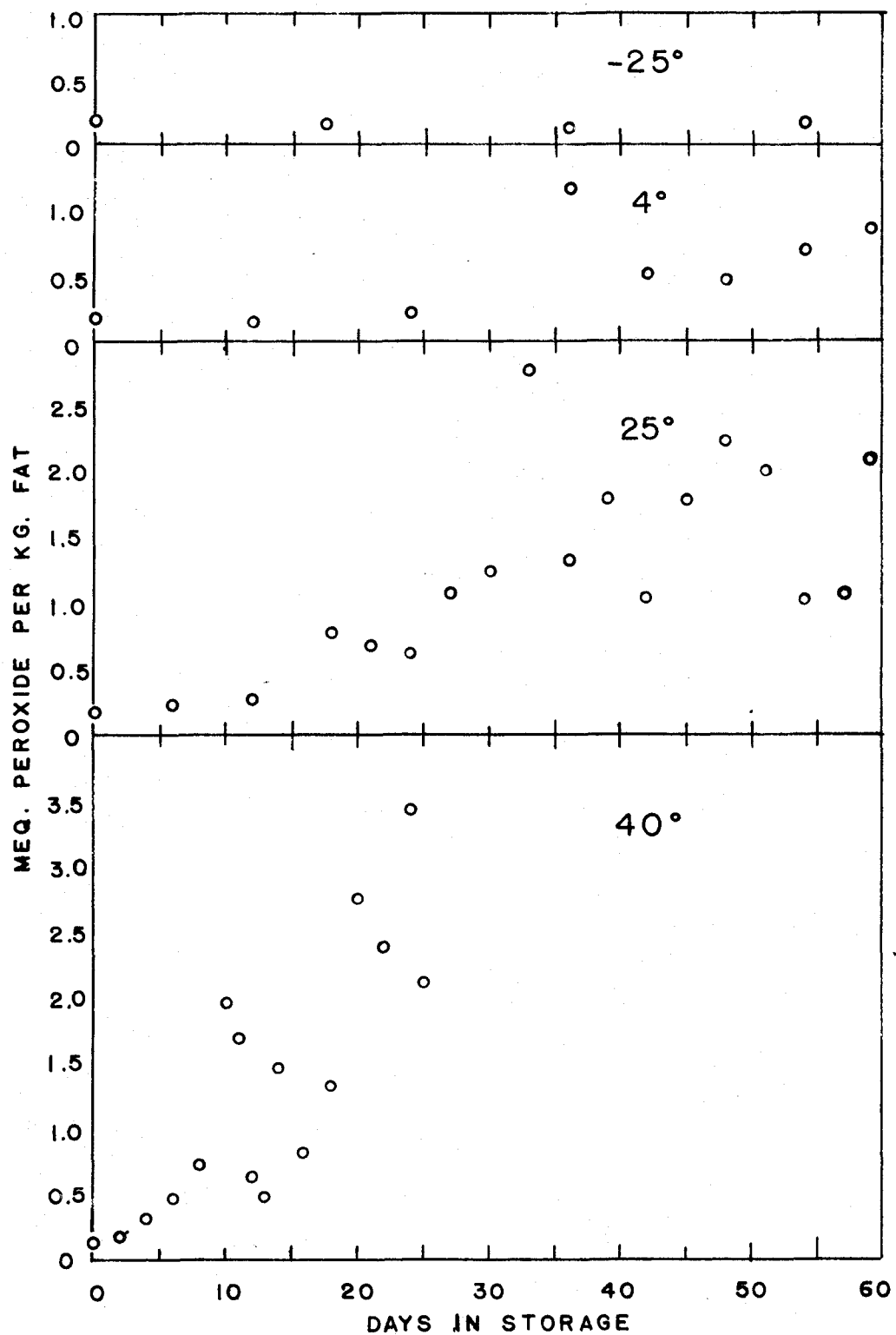


Figure 10. Changes in peroxide content of June fat during storage at various temperatures.

the hydrogenation value becomes greater on oxidation by reason of the peroxide grouping reacting with hydrogen after having formed at a position not previously reacting with hydrogen, this would be expected. The results at other temperatures were not significant except possibly at 4° on the June fat where the coefficient of regression was +0.012.

(4) Carbonyl content. At all temperatures except the lowest, there were positive coefficients of regression of carbonyl content with respect to time. However, the correlation coefficients were variable with regard to significance. When calculated for the entire duration of storage, the correlations for the March fat at 40° and the June fat at 4° were very significant while both fats at 25° were significant and the March fat at 4° was possibly significant. The other results were not significant. The numerical values of the regression coefficients did not show any appreciable variation except that the coefficient for the March fat at 40° was much higher than the others, being more than four times that of the June fat at the same temperature. The data for these relationships are plotted in Figures 11 and 12. Inspection of the plots shows that there may be a slight tendency for the carbonyl content to level off or decrease after approximately 50 days at 25°. No curvilinear regressions were calculated for these or any of the other relationships. It is believed that such regressions would be of value if made on a larger

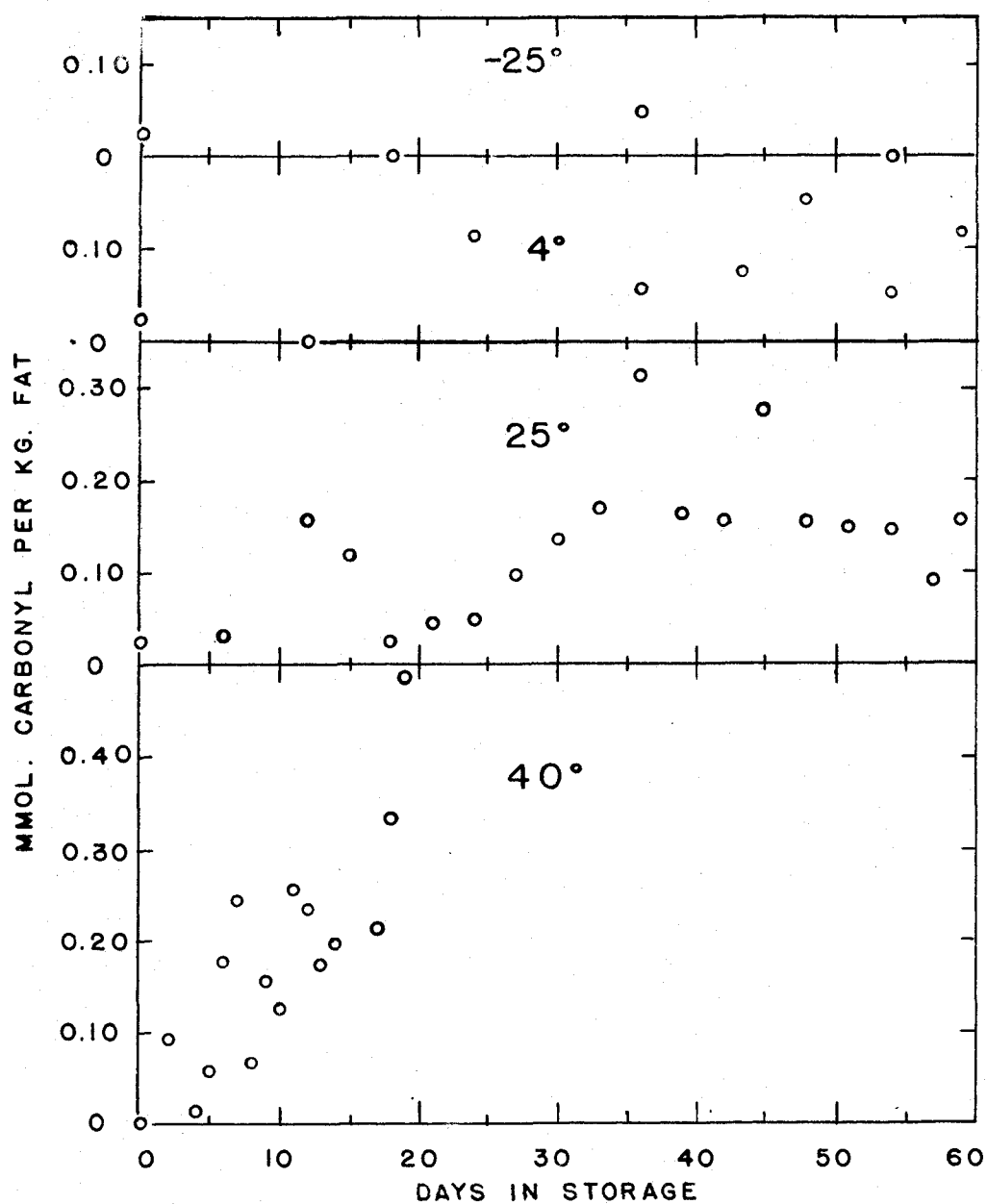


Figure 11. Changes in carbonyl content of March fat during storage at various temperatures.

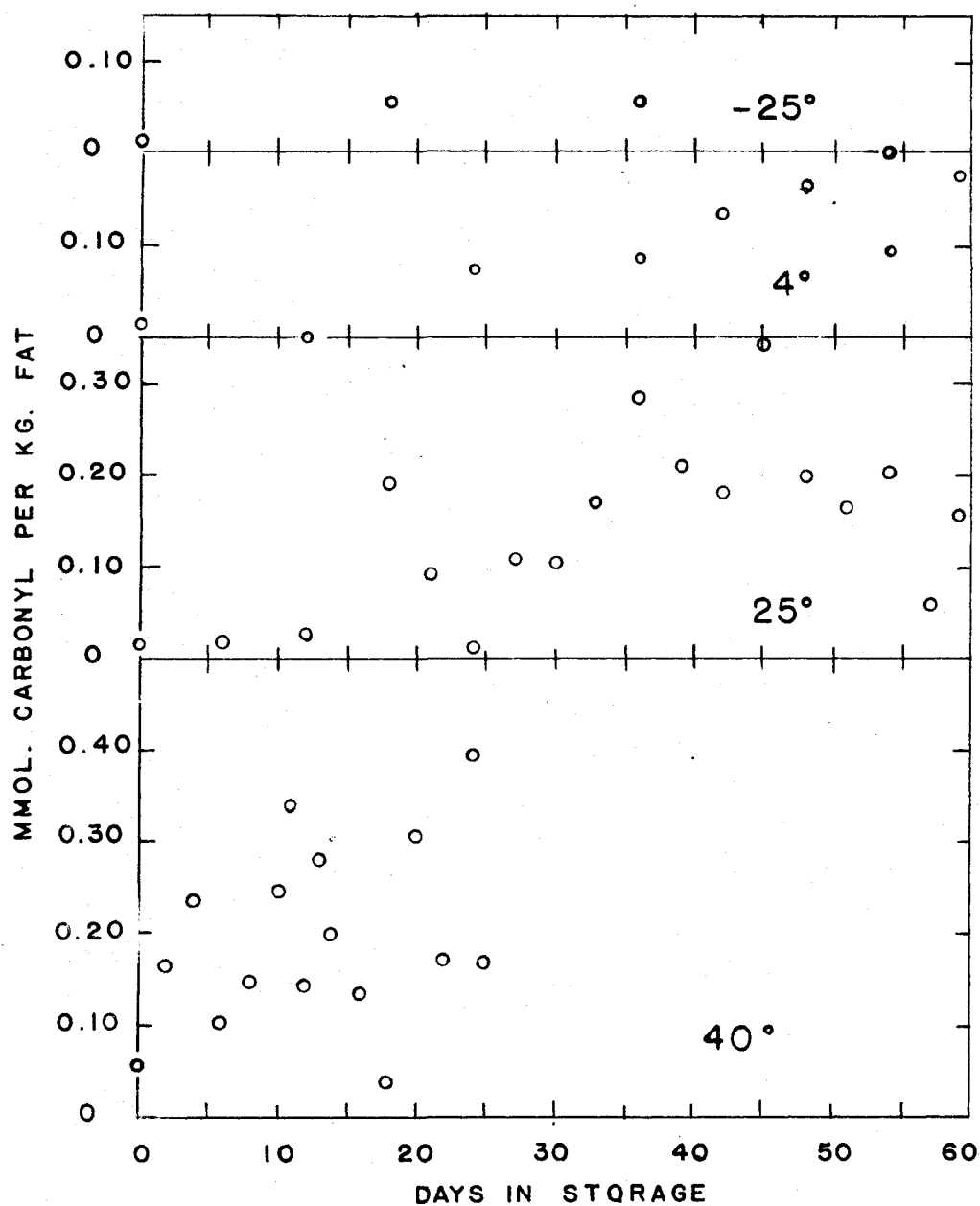


Figure 12. Changes in carbonyl content of June fat during storage at various temperatures.

number of analyses.

(5) Tocopherol content. While at 40° the tocopherol content with relation to time showed a highly significant correlation with the June fat and a significant correlation with the March fat, there was no correlation between tocopherol and time with either fat at 25° . At 4° there was significant correlation with the March fat and possibly with the June fat. The correlation coefficients for the two higher temperatures are negative as would be expected but oddly they are positive for the lower temperatures. The apparent increase in the tocopherol content is quite likely the result of an increase in some reducing compound other than tocopherol as it seems unlikely that tocopherol itself would be formed in the fat in vitro. The nature of this reducing compound is unknown. It may possibly be an aldehyde. This, however, is believed unlikely because it is believed that at the higher temperatures the production of aldehydes proceeds so rapidly that if aldehydes were involved there would continue to be an apparent increase in tocopherol at the higher temperatures rather than at the lower temperatures only. The results obtained in this study indicate the need for further study on the nature of the reactions occurring in fat oxidation and in antioxidative protection.

The results for the samples stored at 40° show a much greater rate of destruction of tocopherol in the March sample

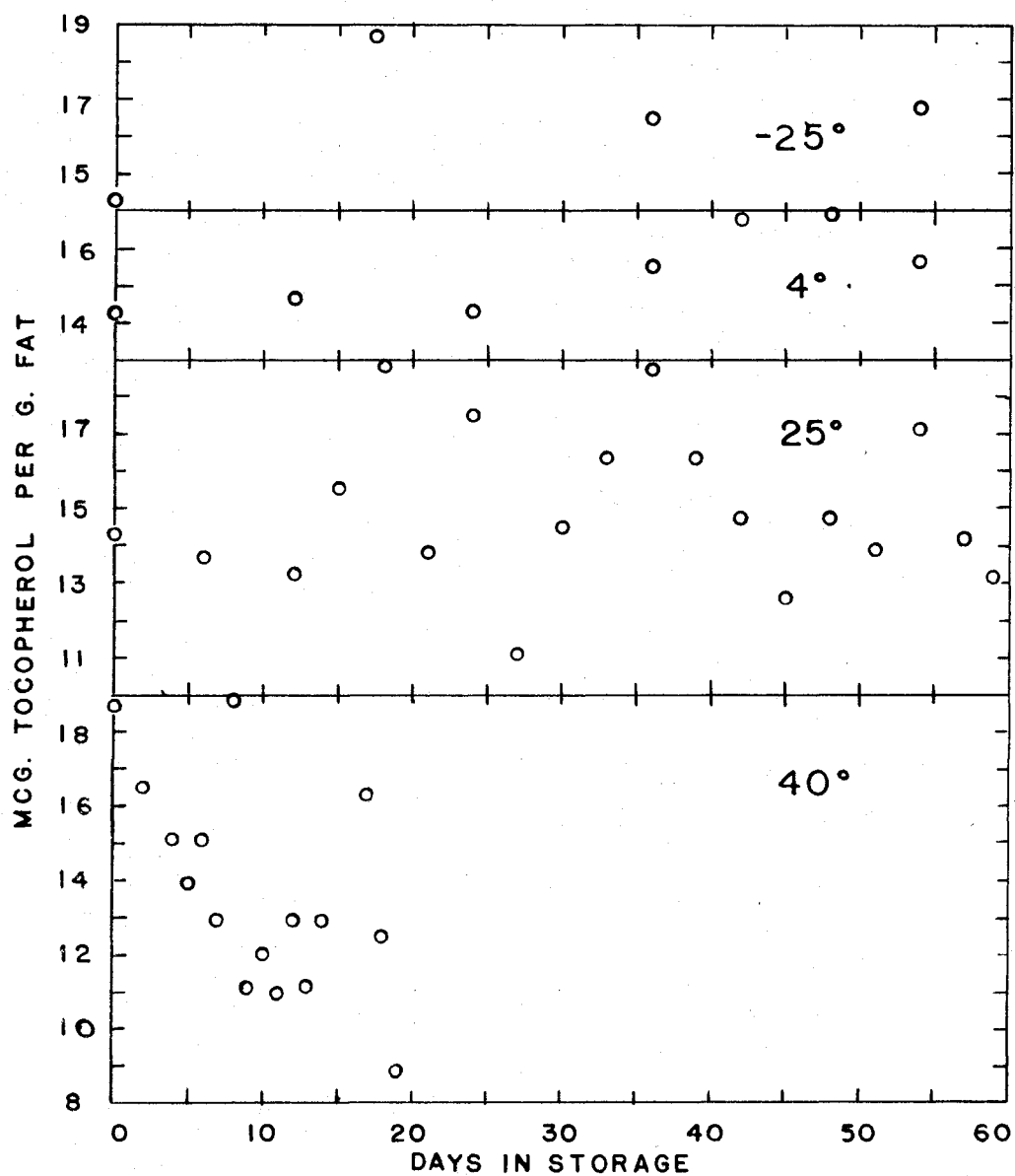


Figure 13. Changes in tocopherol content of March fat during storage at various temperatures.

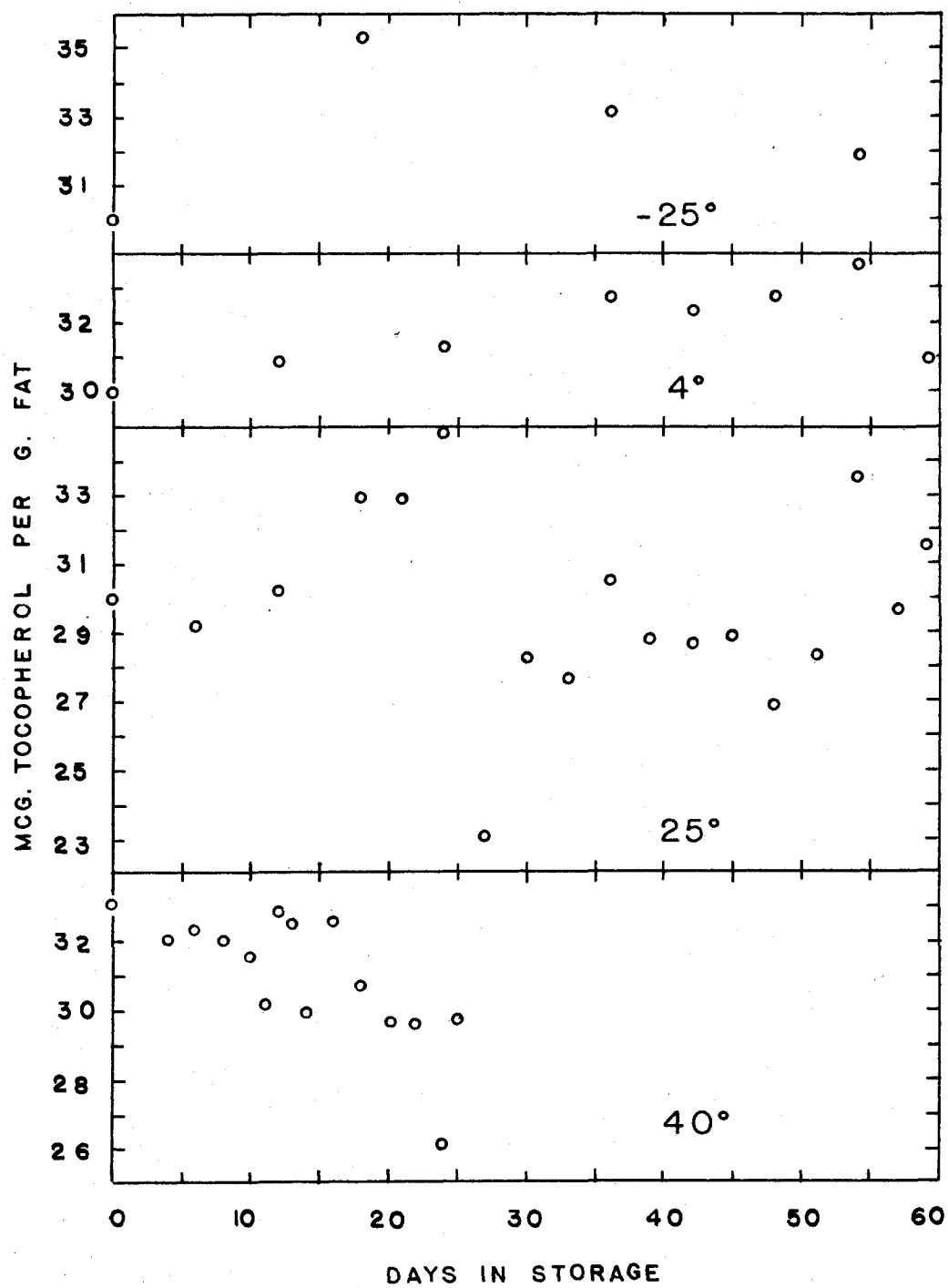


Figure 14. Changes in tocopherol content of June fat during storage at various temperatures.

than in the June fat, the regression coefficients being respectively -0.299 and -0.189 mcg. tocopherol per g. of fat per day. The reason for this difference is not apparent. It had been postulated that perhaps the reason for the greater susceptibility of winter fat to oxidation in spite of its greater saturation was its reduced content of tocopherol as a natural antioxidant. If the results obtained here at 40° can be reproduced, it may mean that some factor other than tocopherol reduction is operative. If so, it remains to be determined whether the tocopherol is oxidized coincidentally with the fat or whether its oxidation permits oxidation of the fat to proceed more rapidly.

(6) Organoleptic flavor score. While the chemical analyses that have been presented indicated a greater rate of change in the March fat than in the June fat, the flavor deterioration occurred at a greater rate in the June fat, which was contrary to expectation. The relationships were very significant on the June fat at all except the lowest temperature and were likewise very significant at 25° on the March fat. The samples of both fats at the other temperatures did not show significance except possibly the March fat at -25° in which case the number of samples was so small that the results would be subject to question. At 25° both fats showed highly significant correlations; the coefficients of regression were -0.054 and -0.142 flavor score units per day for the

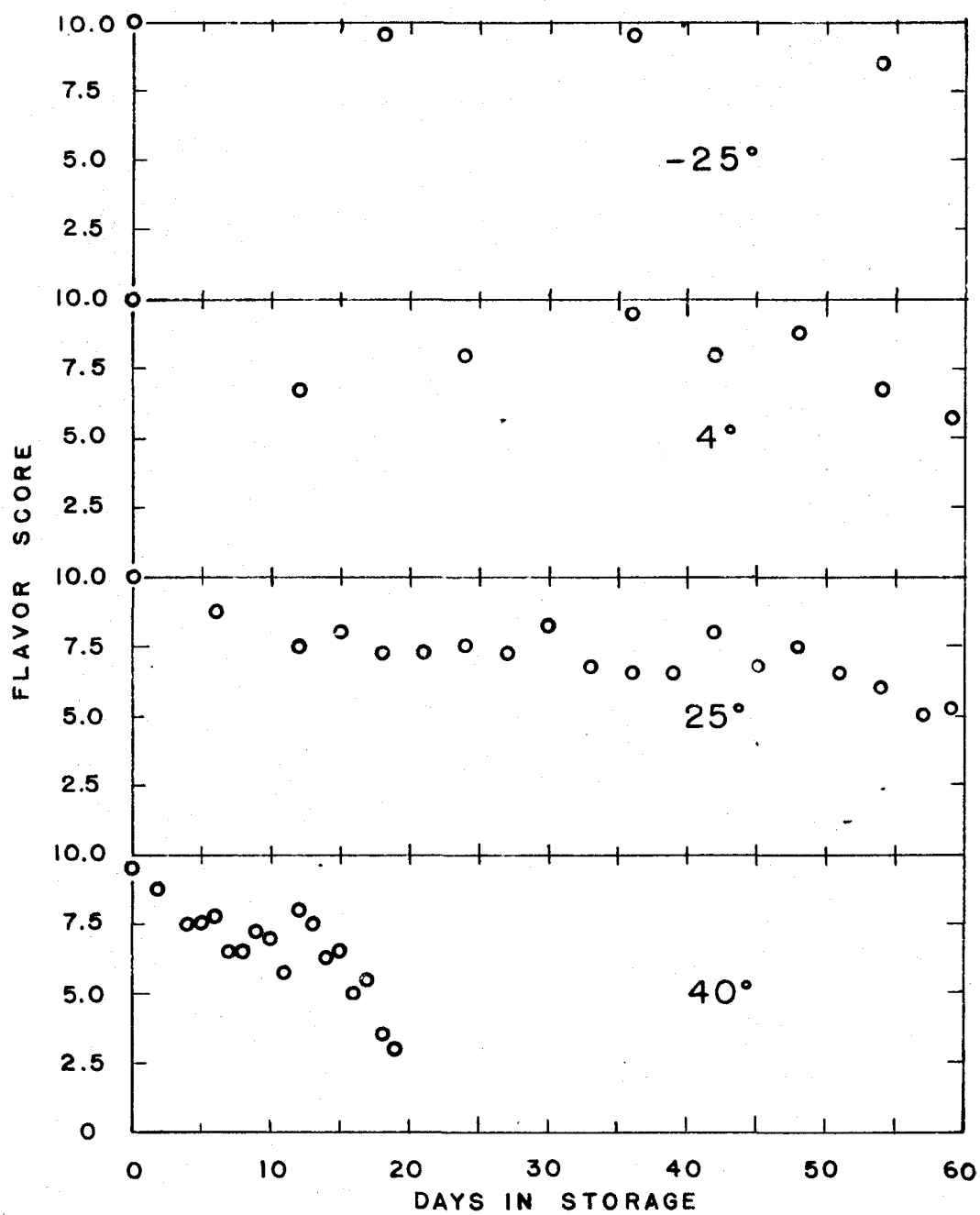


Figure 15. Changes in flavor score of March fat during storage at various temperatures.

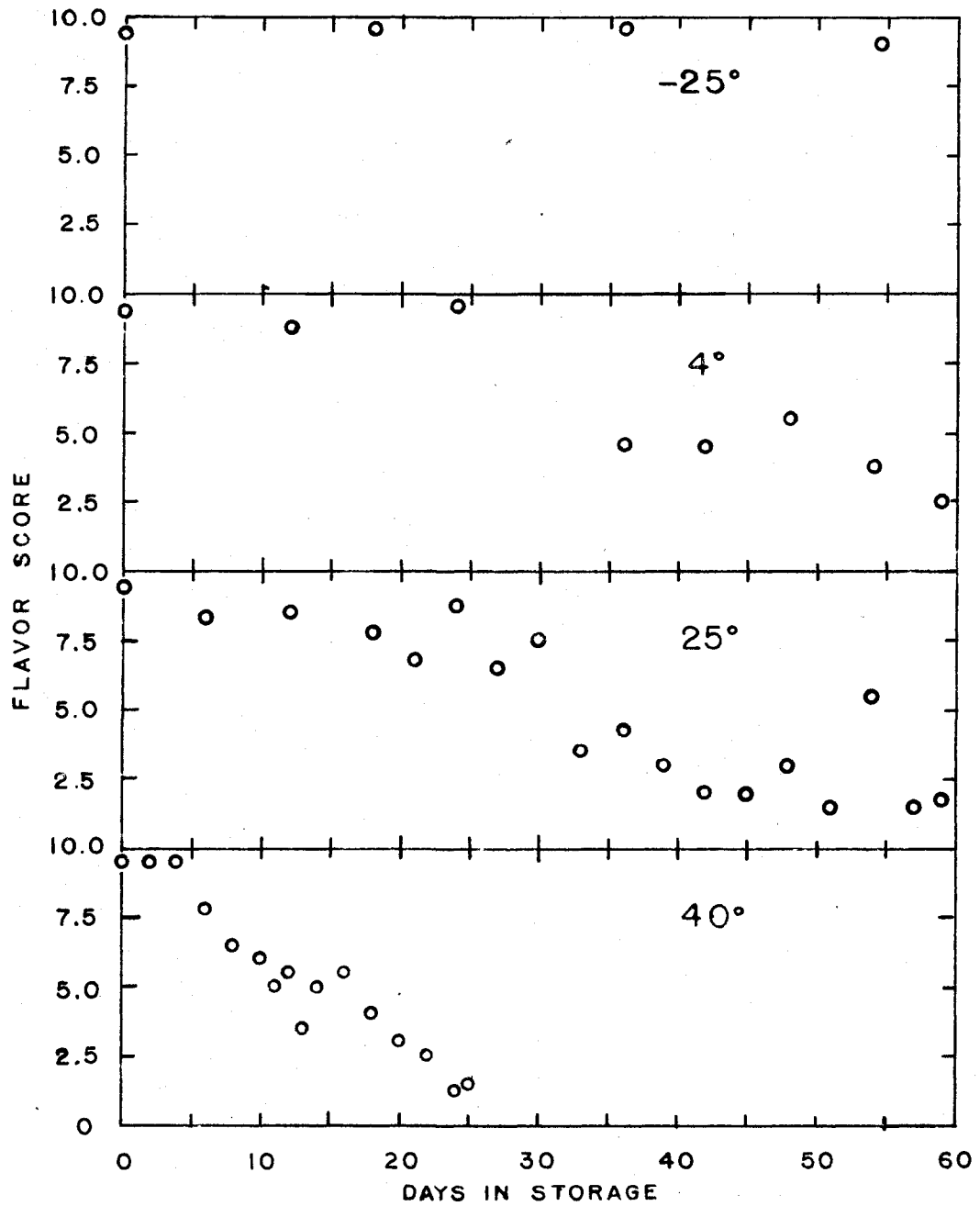


Figure 16. Changes in flavor score of June fat during storage at various temperatures.

March and June fats respectively. The coefficients at 40° were -0.247 and -0.339 respectively; the March figure is not significant (as determined by correlation coefficient).

c. Relationship of other values to organoleptic score

(1) Hydrogenation iodine values. Neither fat showed any significant correlation between hydrogenation value and flavor score at any temperature. However, all regression coefficients were negative, possibly indicating a tendency for the hydrogenation value to increase as flavor score decreased. Disregarding the lowest temperature (where the samples were analyzed only four times) the absolute numerical value of the coefficients on the March fat were all greater than any for the June fat.

(2) Iodine values. The June fat stored at 40° exhibited a significant correlation between the iodine value and flavor score and the March fat at the same temperature showed a highly significant correlation to a flavor score of 6 (13 days) but not for the entire duration of storage (19 days). Otherwise there was a complete lack of significance.

(3) Peroxide content. The March fat shows a very highly significant correlation of peroxide content relative to flavor score at 25° but not at other temperatures when considered for the entire duration of storage. However, at 40° there was a significant relationship to a flavor score of 6 and a highly significant correlation to a flavor score of 7.

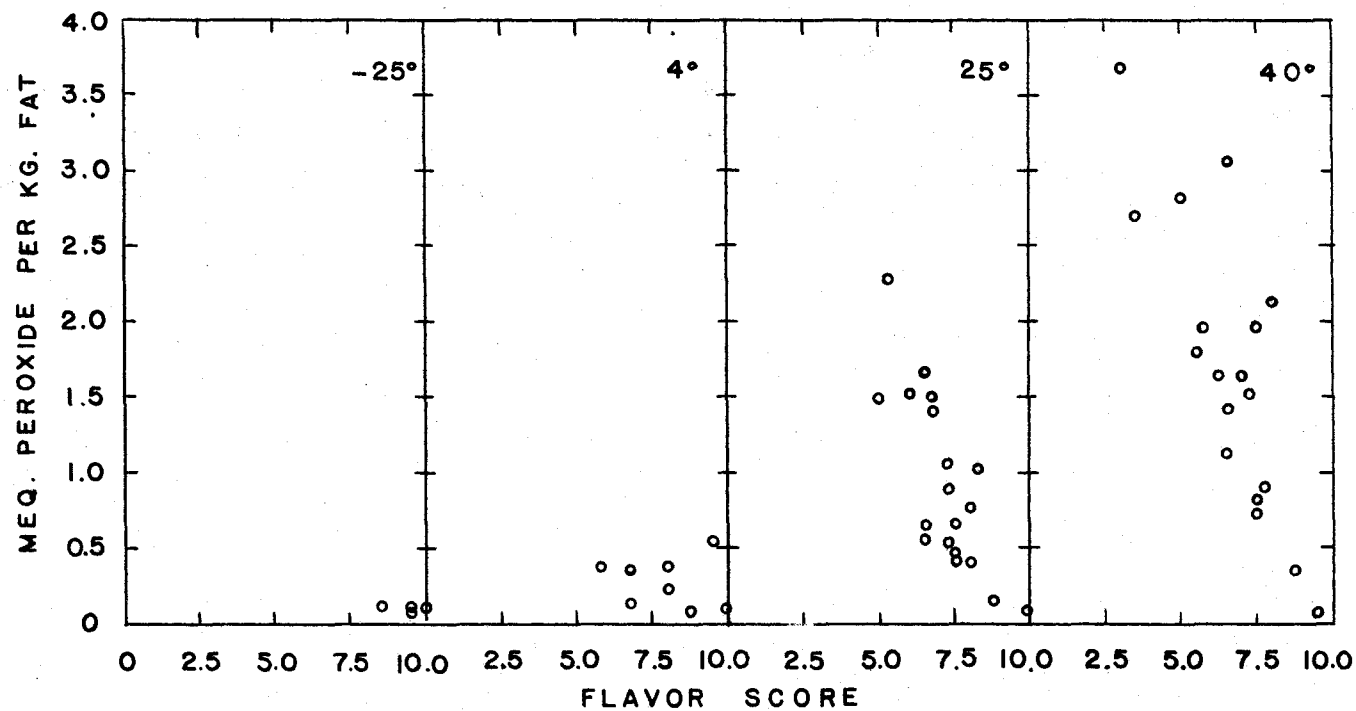


Figure 17. Relationship of peroxide content to flavor score during storage of March fat at several temperatures.

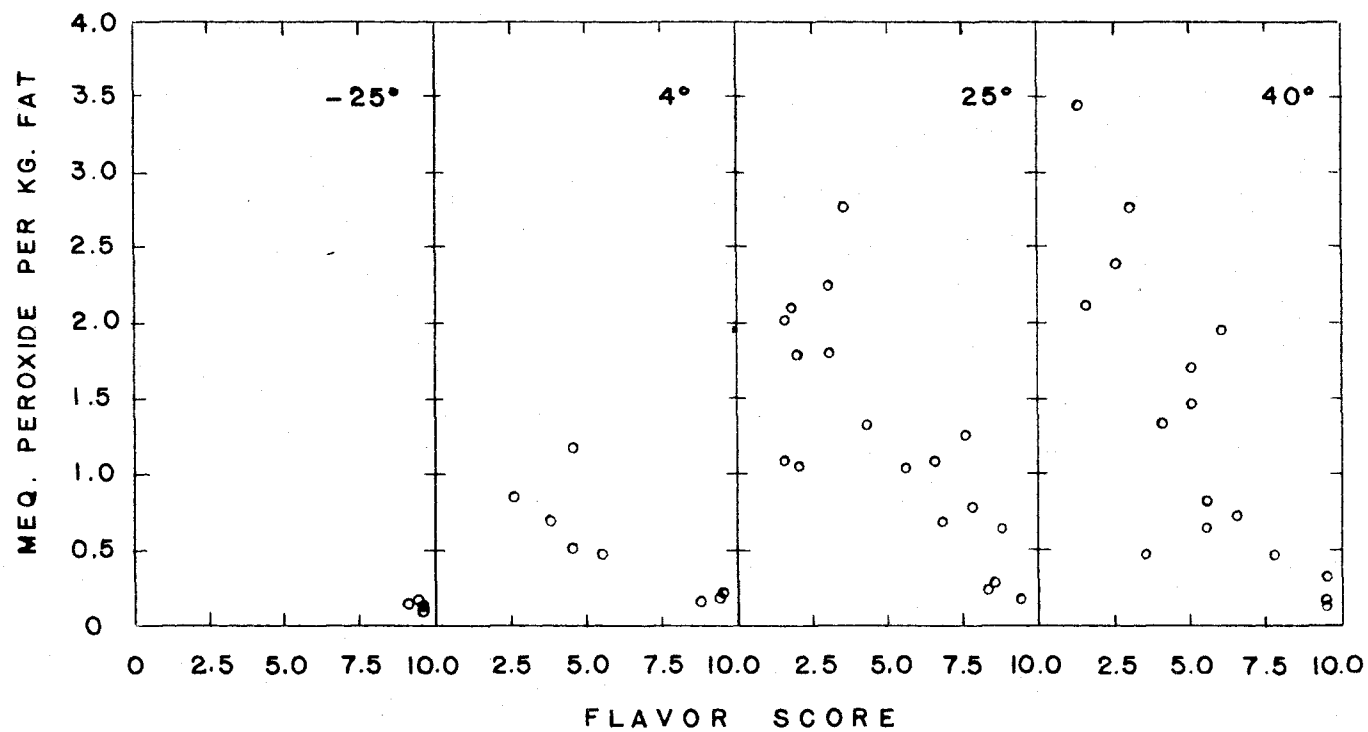


Figure 18. Relationship of peroxide content to flavor score during storage of June fat at several temperatures.

With the June fat the correlations were significant at 4° and very significant at the two higher temperatures. At the two higher temperatures the regression coefficients for the March fat indicate a greater increase in peroxide value for a given decrease in flavor score than for the June fat. The values for the March fat are -0.383 and -0.468 meq. peroxide per unit of flavor score at 25° and 40° respectively, while the corresponding values for the June fat are -0.199 and -0.302.

(4) Carbonyl content. When considered for the entire time of storage, the June fat stored at 4° showed a significant relationship between carbonyl content and organoleptic score and at other temperatures was possibly significant. The relationship with the March fat was possibly significant at 25° but not significant at other temperatures. In all cases except the lowest, the coefficients of regression were negative indicating a tendency toward increase in carbonyl content with decrease in flavor score. This was expected but it is surprising that there is not a higher degree of correlation shown here.

(5) Tocopherol content. Except for a highly significant correlation in the June fat at 40° there was no correlation between the tocopherol content and the flavor score. The regression coefficients were variable in sign although the one significant relationship was positive indicating a decrease in tocopherol with decrease in score.

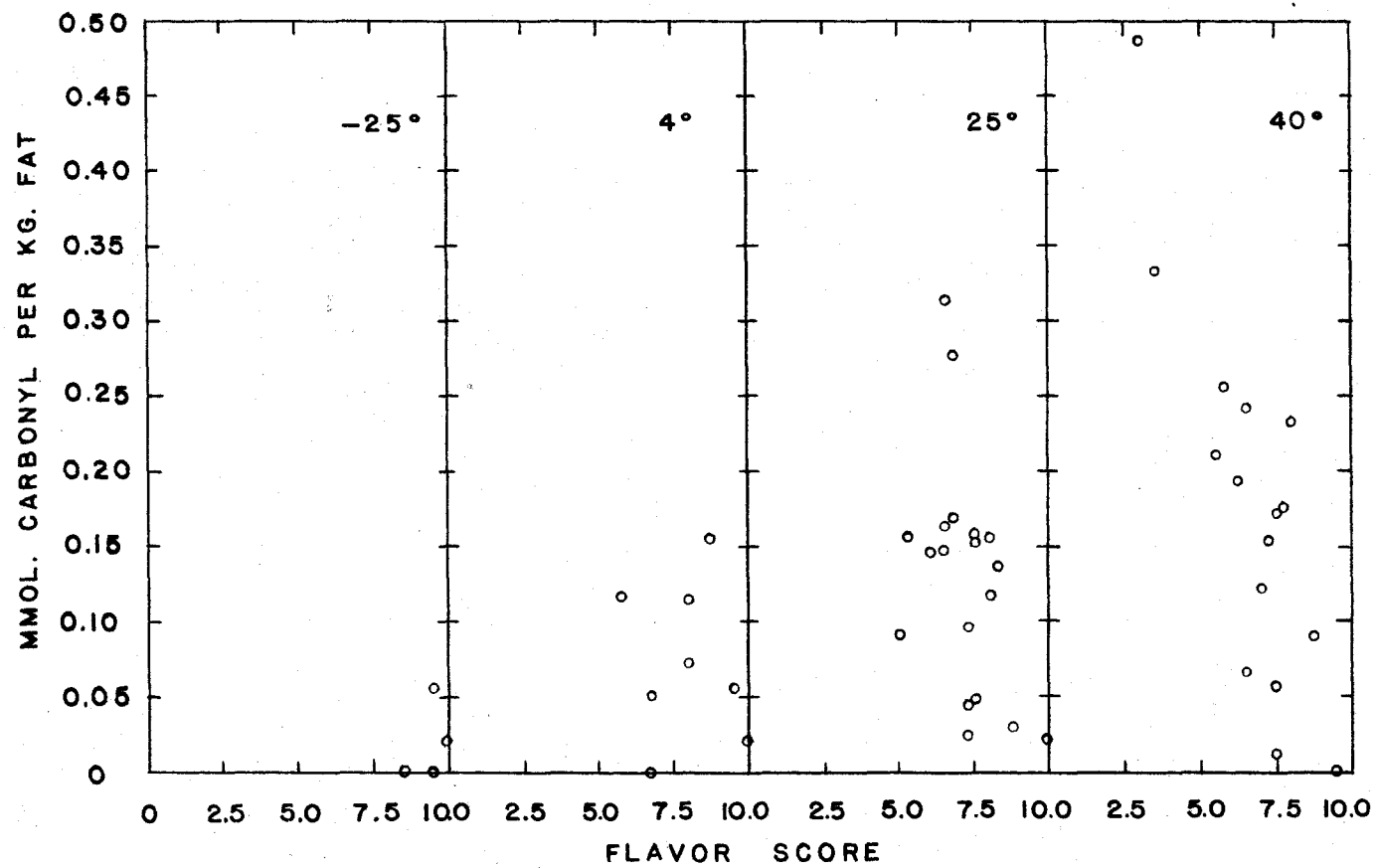


Figure 19. Relationship of carbonyl content to flavor score during storage of March fat at several temperatures.

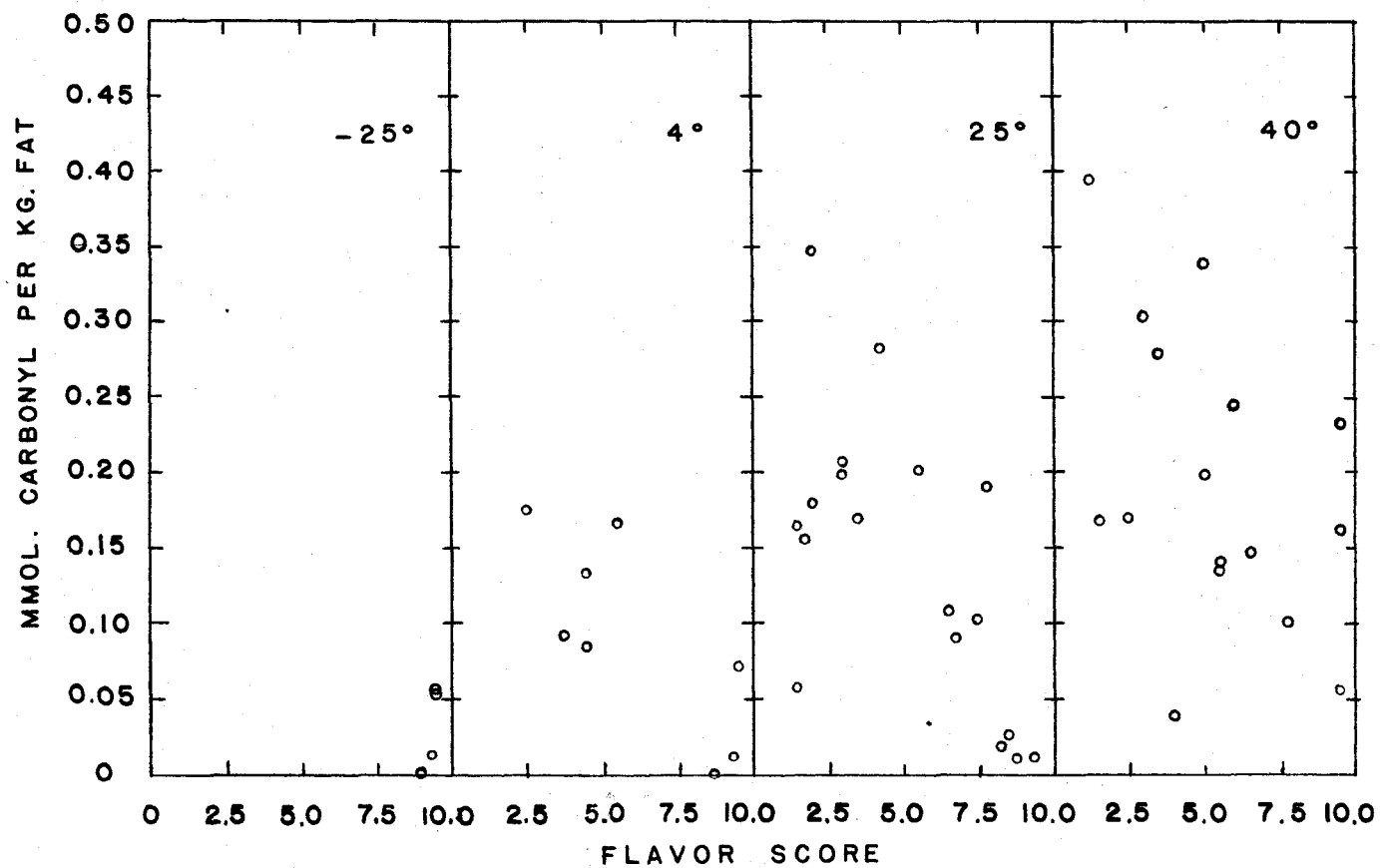


Figure 20. Relationship of carbonyl content to flavor score during storage of June fat at several temperatures.

Apparently, from the lack of correlation, the relationship must be only coincidental. One would, of course, expect both to decrease with time but there is apparently no relationship between their rates of decrease.

d. Relationship between hydrogenation iodine value and iodine values. As was previously pointed out, there was noticed during the development of the hydrogenation method an ever present difference between the hydrogenation iodine value and the iodine value. This difference was not always of the same magnitude with different fats. The same type of difference was evident in the studies on the oxidizing fats. With the March fat, the overall average of all the hydrogenation values exceeded the average of all iodine values by 0.39 units while with the June fat the difference amounted to 1.37 units. Some of the implications of this difference were discussed on page 173.

Both fats when stored at 40° and the June fat when stored at 25° showed significant correlations between the hydrogenation and iodine values while all other samples showed no correlation at all. Peculiarly, though, the March sample had positive coefficients of regression at -25° (only four analyses) and 40° . The latter series is one which had shown a non-significant increase in iodine value with time when all others in the group were decreasing. This same series showed a negative coefficient regarding the regression of the difference

between these two analytical values against time whereas all others were positive for this regression. For both the regression of hydrogenation value against iodine value and the regression of the difference against time, the magnitudes of the coefficients seemed to be independent of temperature. Therefore, it is somewhat doubtful whether this difference is a function of oxidation.

e. Effects of temperature.

(1) On iodine values. While the magnitudes of the regression coefficients of iodine value against time do not give any definite indication of the effect of temperature, there does seem to be a greater tendency toward significance in the correlations at higher temperatures. The same comments would apply to the relationship between iodine values and organoleptic flavor scores as well as between hydrogenation value and iodine value.

(2) On peroxide development. Both the March and the June fats at 25° and at 40° show highly significant correlations between peroxide content and time, with the March fat developing the peroxide more rapidly. Taking the rate of peroxide formation for each fat at 25° as unity, the rate at 40° is 6.00 and 3.66 for the March and June fats respectively. The correlations at other temperatures are not significant except possibly for the June fat at 4° where the rate of formation is approximately 0.4 that at 25°. Judging by

correlation coefficients, there may possibly be a tendency toward more significant correlation between peroxide content and flavor score at the higher temperatures. In fact for the June fat there is no correlation at -25° , a significant correlation at 4° and very significant correlations at 25° and 40° . The correlation of peroxide with tocopherol content shows no significance except at 40° where it is very significant for both fats.

(3) On carbonyl development. With the March fat the degree of significance of the correlation of carbonyl content with time steadily increases with increasing temperature from no correlation at -25° to highly significant correlation at 40° . There appears to be little difference in the rate of carbonyl development with increase of temperature from 4° to 25° but (judging by the March fat where the correlations are significant or better) an increase from 25° to 40° causes more than a seven-fold increase in the rate of carbonyl development.

(4) On rate of flavor deterioration. If, on the June fat where the correlations are very significant except at -25° , one considers the rate of decrease in flavor score at 4° as unity, the rate at 25° is 1.18 and that at 40° is 2.82. While the data here, for lack of significance do not allow an exact comparison on the March fat, there are indications that the temperature coefficient on increasing the temperature to 40° would be at least as high as for the June fat.

(5) On relationship of iodine and hydrogenation values. While temperature appears to have little effect on the magnitude of the regression coefficients of hydrogenation value versus iodine value or of the ratio of these two analytical values against time, there appears to be increasing significance in the correlation coefficients as the temperature increases. There is significance in the correlations for both fats at 40° and for the June fat at 25°.

(6) On tocopherol content. The coefficients of regression of tocopherol against time are very interesting in that, at the two lower temperatures, the values are positive, those at 4° being almost twice those at -25° (though the correlations at -25° are not significant). For both fats at 25° the correlations are not significant but the regression coefficients are negative and of low magnitude. At 40° the coefficients of regression are also negative but have increased considerably in magnitude. Since it appears unlikely that tocopherols would be formed in the fat in vitro, some substance in the fat not normally reacting as tocopherol in the tocopherol procedure must be converted to a substance which will react as tocopherol. At the lower temperatures this must occur at a greater rate than the destruction of the tocopherol while at some temperature apparently between 4° and 25° the two rates must become equal.

f. Differences between summer and winter fats. The magnitude of the analytical values indicate certain initial differences between the two fats quite apart from their behavior in storage, although the behavior in storage is considered dependent upon these differences to some extent. The initial tocopherol content of the June fat was slightly more than twice that of the March fat, the values being 30.01 and 14.24 mg. per g. fat respectively. If tocopherol does in fact act as an antioxidant in butter fat, this would tend to make the summer fat more stable toward oxidation, in conformity with actual observations in the market milk industry. In addition to a greater initial tocopherol content the results show a less rapid rate of breakdown of tocopherol in the June fat. In agreement with the idea that the smaller tocopherol content and more rapid tocopherol degradation should make the March fat more susceptible to oxidative deterioration, the peroxide and carbonyl contents were found to increase more rapidly in the March fat. The hydrogenation value also increased more rapidly in the winter fat. In spite of all these indications that the March fat deteriorated more rapidly than the June fat, the flavor score of the June fat dropped the more rapidly. From the standpoint of the degree of unsaturation alone, this is as would be expected since the June fat showed roughly 20 per cent more unsaturation. This raises the question of whether peroxide and carbonyl

production might be a function of either the initial tocopherol content or its rate of degradation while the flavor deterioration is a function of the degree of unsaturation. It would seem likely that if flavor deterioration were a function of the degree of unsaturation and if the flavor deteriorated more rapidly in the June fat, this fat should show a greater rate of change in the hydrogenation iodine value or the iodine value. Such is not indicated by the results obtained. One might suggest that flavor deterioration is a resultant of two or more opposing factors. Actually, this seems more logical than that it should be a function of only one factor. But since all the results except flavor score showed a greater rate of change in the March fat (or no significant difference) there must undoubtedly be some factor at play which is not indicated by the chemical analyses.

Although the March fat showed the greater rate of accumulation of peroxides and the greater rate of degradation of tocopherols, the June fat showed a much greater peroxide accumulation per unit of tocopherol degraded. At 40°, where the figures are either significant or highly significant, the peroxide accumulation and tocopherol degradation occur about 1.5 times as fast in the March fat than in the June fat while the June fat shows about twice the amount of peroxide per unit of tocopherol degraded than is shown by the March fat. If one assumes, on the basis of the hypothesis of alpha-methylene

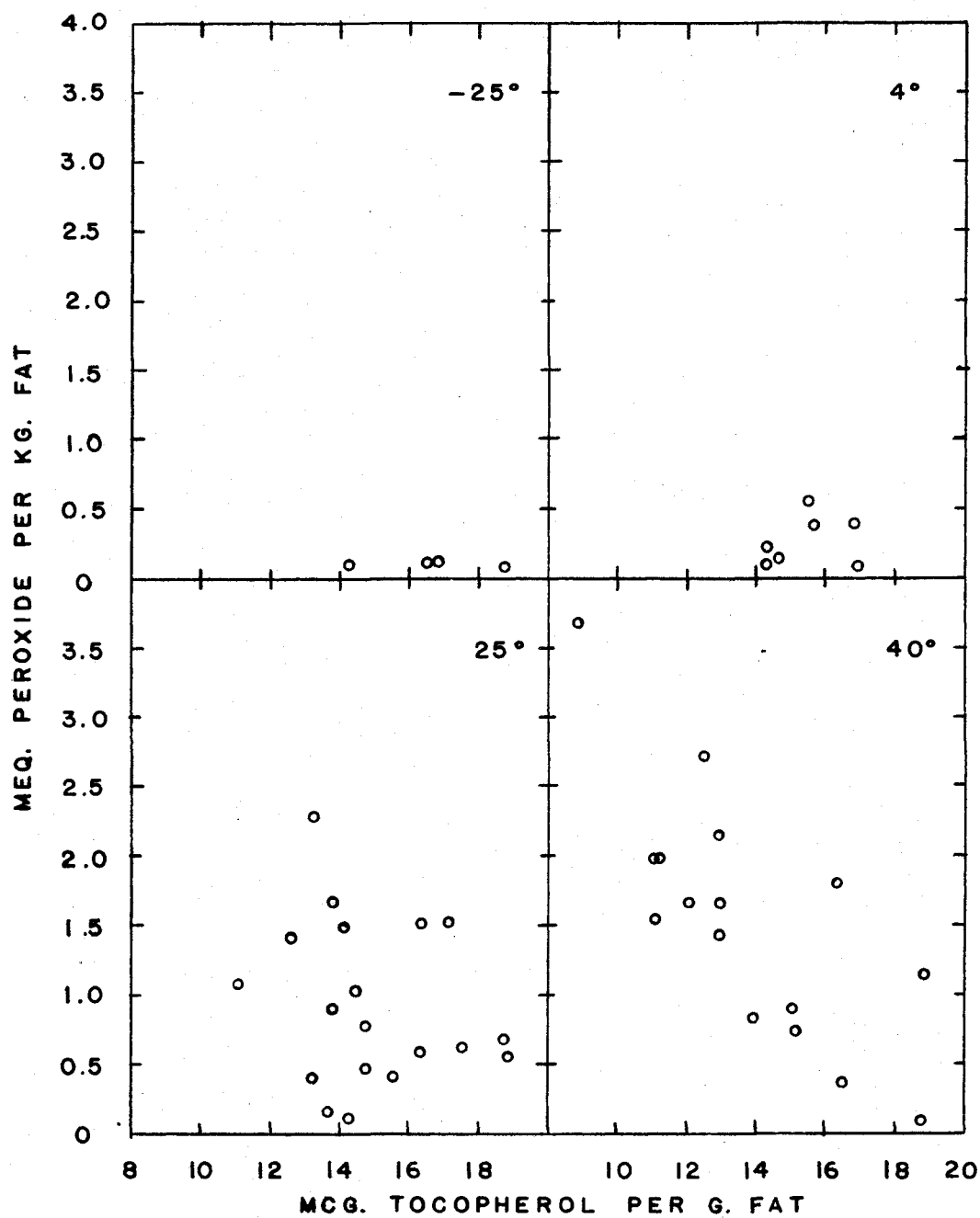


Figure 21. Relationship of peroxide content to tocopherol content during storage of March fat at several temperatures.

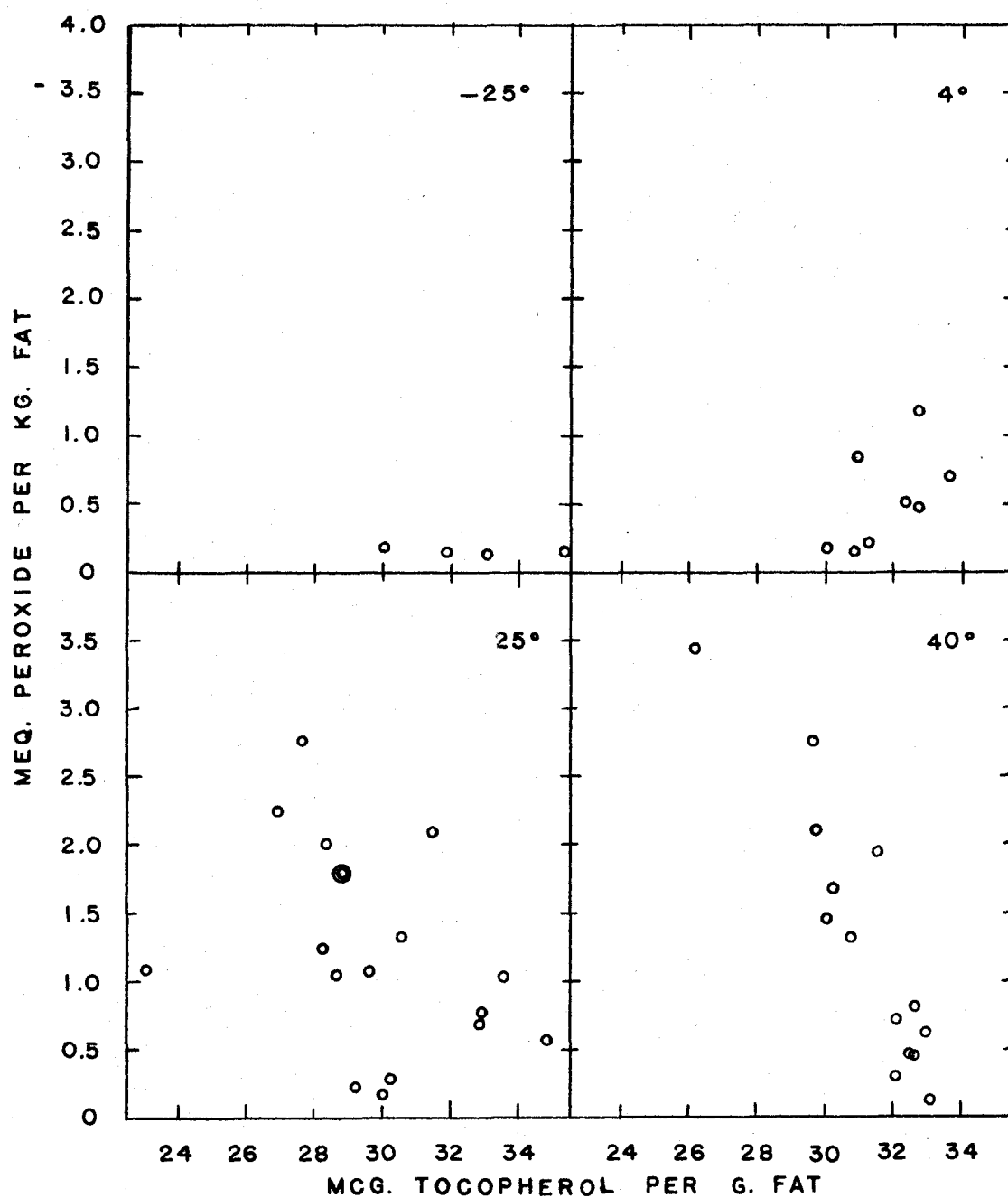


Figure 22. Relationship of peroxide content to tocopherol content during storage of June fat at several temperatures.

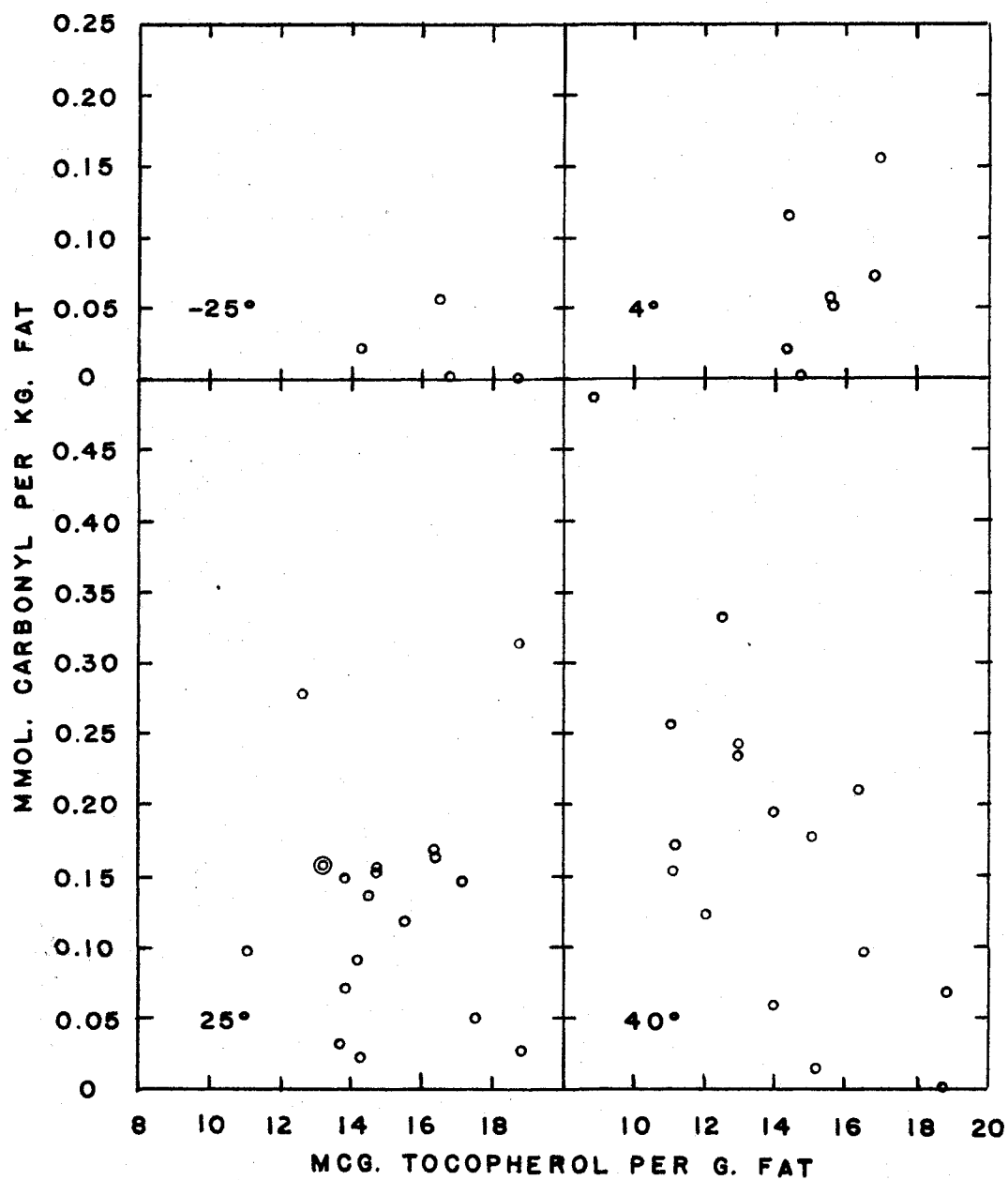


Figure 23. Relationship of carbonyl content to toco-
pherol content during storage of March
fat at several temperatures.

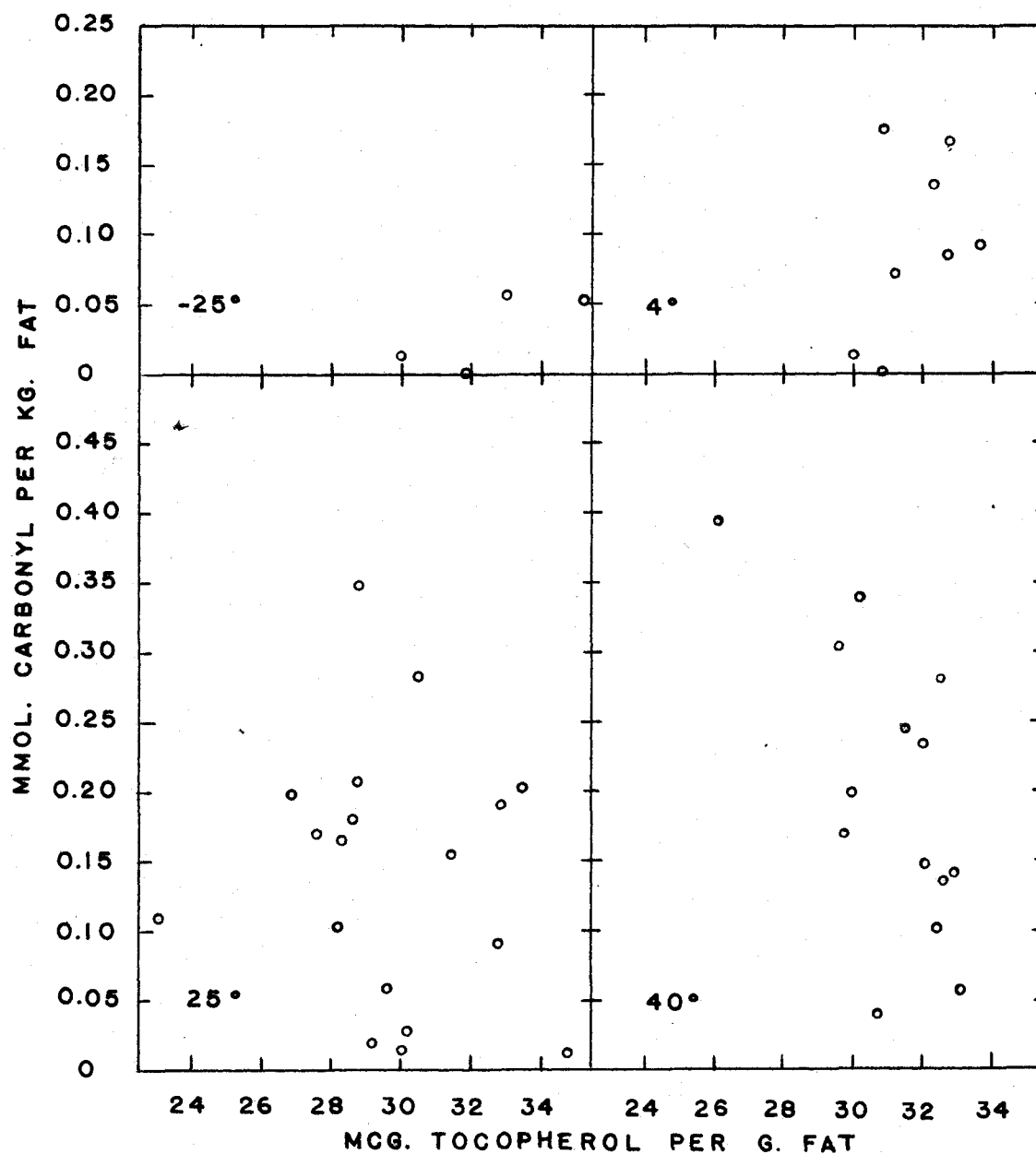
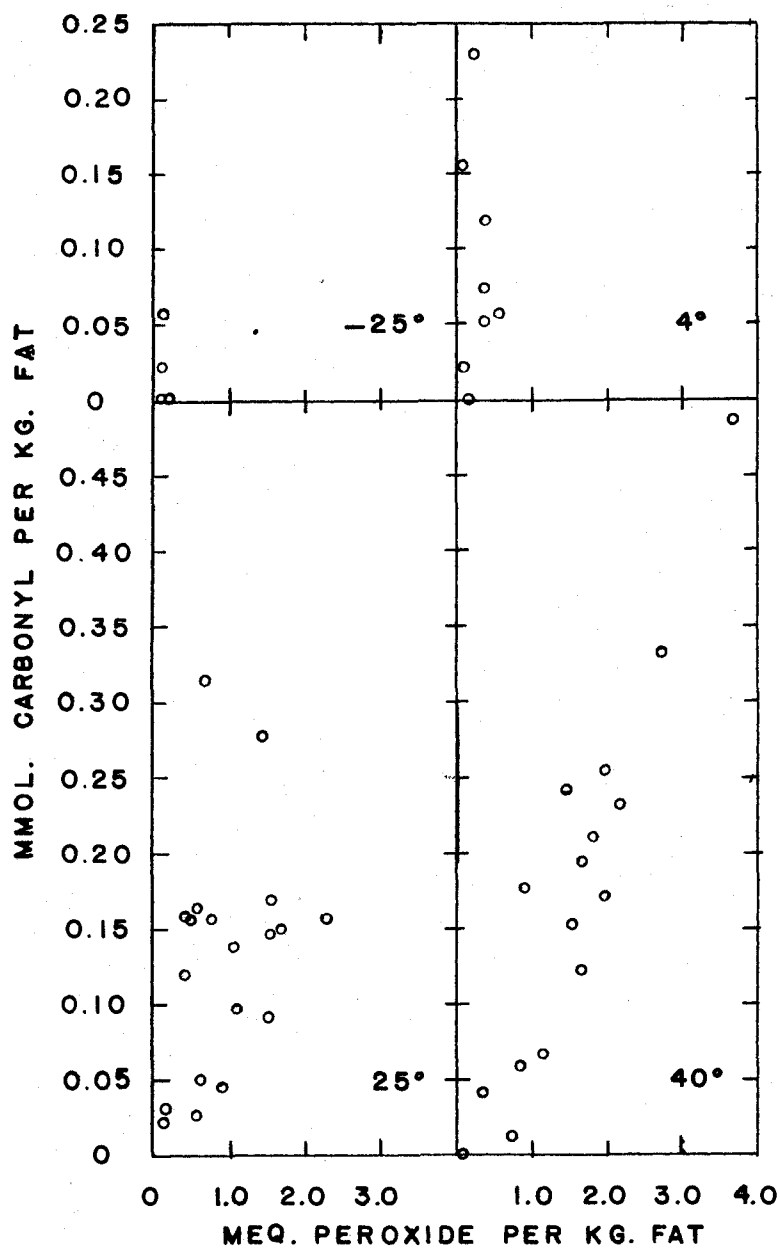


Figure 24. Relationship of carbonyl content to tocopherol content during storage of June fat at several temperatures.



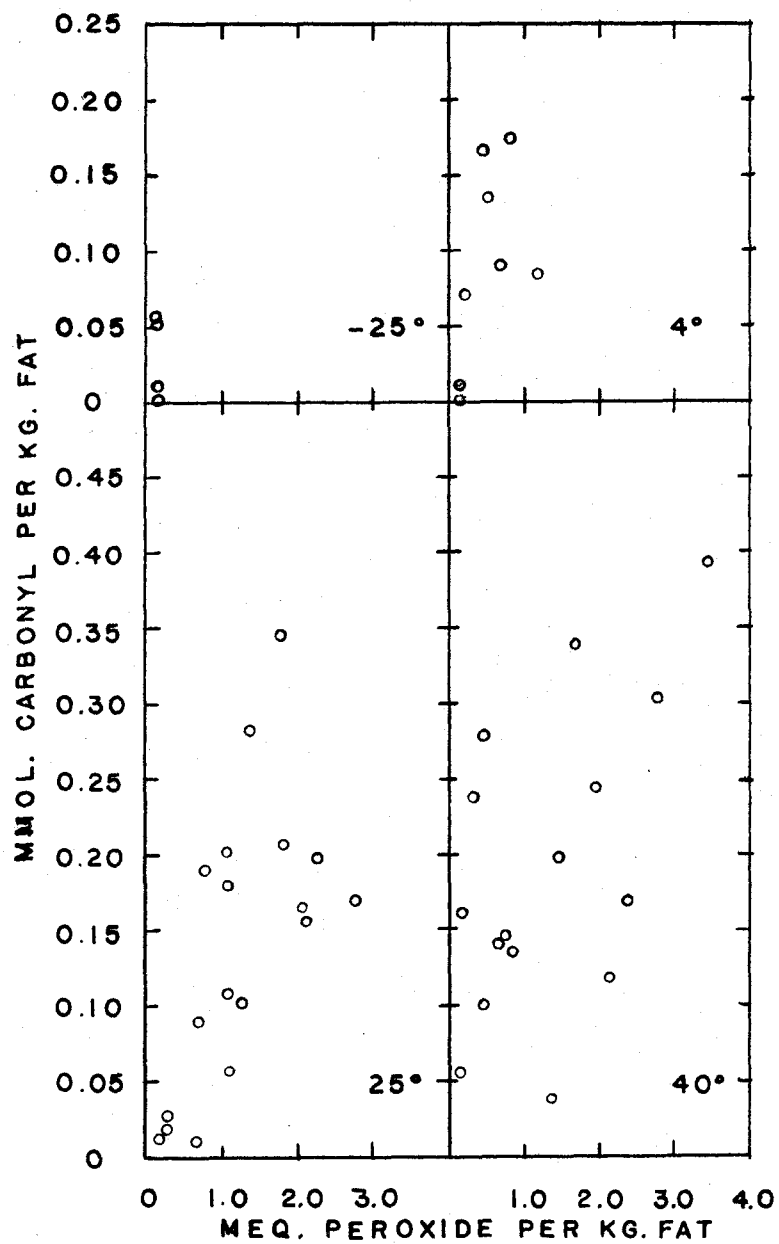


Figure 26. Relationship of carbonyl content to peroxide content during storage of June fat at several temperatures.

reactivity and chain mechanism, that the amount of tocopherol degraded is directly related to the number of chains broken, this would indicate that in the winter fat, more chains are broken in a given time than in the summer fat. With the additional assumption that the amount of peroxide accumulated is related directly to the number of reactions occurring, these data would seem to indicate that in the summer fat there are more reactions per chain.

g. Hypotheses concerning development of oxidized flavor.

On the basis of the above considerations, two alternative hypotheses concerning the development of oxidized flavor have been developed.

It is believed that free radicals may take part in three types of reactions, namely (a) reactions producing peroxides (and by decomposition of these, carbonyl compounds), (b) chain termination reactions related to tocopherol oxidation, and (c) reactions producing compounds responsible for oxidized flavor.

If the number of chain termination reactions is directly proportional to the amount of tocopherol oxidized, the production of more peroxides per unit of tocopherol oxidized must be the result of longer oxidation chain length. If the percentage of the free radicals reacting to form peroxides should remain the same it follows that the total percentage taking part in other reactions must remain the same. Therefore when

the percentage taking part in chain termination reactions is decreased as in longer chains some other chain propagating reaction not producing peroxide must occur. It is conceivable that this non-peroxide chain propagating reaction could be responsible for the development of oxidized flavor.

By letting P represent the reactions forming peroxide, F those forming flavor compounds and T the termination reactions of the type involving tocopherol this can be represented as follows:

PPPP F PPPP F PPPP T (for winter fat) and

PPPP F PPPP F PPPP F PPPP F PPPP T (for summer fat).

From these we can see that in the March fat there are two^a flavor reactions and 12 peroxide reactions for each termination while in the June fat there are four flavor reactions and 20 peroxide reactions for each termination reaction. These figures would indicate that the March fat should have a greater amount of peroxide formation and a greater amount of tocopherol degradation for a given amount of oxidized flavor developed than would the June fat. They would also indicate that the June fat should show a greater amount of peroxide formation for a given quantity of tocopherol oxidized. While the results obtained are not conclusive enough to prove the

^aAll figures mentioned in this paragraph and the one following were selected merely for ease of illustration and do not necessarily represent true ratios.

correctness of these assumptions the data are in agreement with these ideas.

If one assumes the same rate of chain initiation in both fats then the above chains would give an equal rate of tocopherol oxidation in both fats but a greater rate of peroxide and oxidized flavor development in the summer fat. Neither the observed tocopherol degradation rates nor the observed peroxide formation rates are in agreement with this. However, it is possible with different rates of chain initiation to have chains such as those represented above which will give results in agreement with the observed rates. For example, if there were to be 11 chains initiated in the winter fat for each six in the summer fat there would be the following ratios in the reactions:

132 P : 22 F : 11 T (winter fat) and

120 P : 24 F : 6 T (summer fat).

This would indicate a greater rate of production of oxidized flavor in the summer fat but greater rates of peroxide formation and tocopherol oxidation in the winter fat. These relationships are the same as those observed with the March and June fats studied.

Many assumptions have been made in arriving at the above hypothesis but nothing has been found in the literature indicating that the assumptions are invalid. While the figures listed above are selected merely for convenience of

illustrating a point and would not necessarily be those which might be determined by further experiment, they do serve to show that it is possible to have relationships among the three types of reactions such as to account for the observed results on dry fats in terms of the suggested hypothesis. This hypothesis has not as yet been extended to account for the more rapid development of oxidized flavor in winter milk than in summer milk as observed in the market milk industry.

It is suggested that the material which is the chain propagating flavor forming compound may be polyunsaturated materials reacting through hydrogen acceptor-hydrogen donor stages with double bond shift to conjugated unsaturation.

An alternative to the above hypothesis, if one wishes to assume that chain length remains the same in both fats, is that there are two types of chain termination reactions, one involving formation of flavor compounds and the other involving tocopherol oxidation but no flavor compounds. These, using the same symbolism as above, may be represented as



The relative rates of occurrence of these two reactions could be such as to give the same results as the previously mentioned hypothesis.

If this latter hypothesis is valid, the flavor compound may be either the result of interaction of two free radicals or a compound which is oxidized in the process of chain

termination.

The above hypotheses have been based on the assumption that tocopherol is involved in and directly related to the chain termination reactions. This in turn is based upon the hypothesis that tocopherol functions as an antioxidant through the mechanism of chain termination. It is quite possible that tocopherols either are not involved in chain termination or are involved in other reactions in addition to chain termination. In this case the assumptions regarding chain length would not necessarily hold. The hypothesis of the flavor forming reactions and the reactions involving tocopherol being competitive would not be invalidated by this. It may be that there is more than one type of peroxide and that the tocopherol degradation is not related to all types.

h. Effect of storage temperature. If thermal acceleration of oxidation by high temperature storage is to be of value in studying the oxidation occurring at lower temperatures the reactions must be unchanged except for differences in rate. Furthermore the increase in rate must be uniform for all reactions in order that the relative ratios of products formed may be the same. Table 22 gives the relative rates of change of some of the analyses, with the rate of change at 40° taken as unity. Actually some of the figures used in calculating these values were coefficients of regression in cases where the correlation was not significant. As could be seen

Table 22

Relative Rates of Change of Analytical Values^a

| Dependent variable ^b | Direction of change | March fat | | | June fat | | |
|---------------------------------|---------------------|-----------|------|----------------|----------|------|------|
| | | 4° | 25° | 40° | 4° | 25° | 40° |
| Hydrogenation value | increasing | 1. | 0.83 | 4.08 | 1. | 2.00 | 3.83 |
| Iodine value | decreasing | 1. | 0.67 | x ^c | 1. | 1.19 | 3.88 |
| Peroxide content | increasing | 1. | 6.21 | 37.26 | 1. | 2.50 | 9.17 |
| Carbonyl content | increasing | 1. | 1.44 | 11.25 | 1. | 1.11 | 1.54 |
| Flavor score | decreasing | 1. | 1.52 | 6.91 | 1. | 1.18 | 2.82 |

^aValues at 4° taken as unity. Relative rates based on regression coefficients shown in Table 31.

^bTime as the independent variable

^cIncreasing value

by referring to Table 31, in only one case (that of flavor score in the June fat) did the values at all three temperatures show significance. The values for tocopherol content are not shown since they appeared to increase at 4° but to decrease at 25° and 40°. It may easily be seen that these thermal coefficients are not the same for all reactions and therefore it would appear that the reactions do not follow the same course at all temperatures. A study of the significance of the coefficients of correlation of the various analyses would indicate the same thing. In no case is there tendency

to show correlation at low temperatures when there is no correlation at higher temperatures. The reverse, however, is not true. In a number of cases there are correlations at high temperatures but not at low temperatures. The peroxide content shows a very definite trend toward correlation with carbonyl and tocopherol contents, flavor score and time at the highest temperature, in some cases at 25° and almost no significance at 4°. There is a highly significant correlation between carbonyl content and tocopherol content at 40° but no correlation at other temperatures. There are significant correlations between iodine and hydrogenation iodine values of the June fat at 25° and both fats at 40° but otherwise no correlation between these measurements. It is true that not all the samples were held in storage for a length of time sufficient to give the same degree of deterioration in all and therefore some additional study may be indicated. However, the correlation coefficients to similar degrees of oxidation show little in the way of definite trends as regards their significance.

1. Evaluation of various methods of analysis. Organoleptic observations still remain the most reliable criterion of deterioration of a fat. So long as the material retains a pleasing flavor it does not matter to the consumer what the carbonyl, peroxide, or tocopherol contents may be. Other than attempting to discern the course of the oxidation reactions

the reasons for measuring these other values are the desire for a more objective measure of deterioration and the endeavor to find a method of predicting impending deterioration at an earlier stage than is possible with organoleptic observations. Regardless of which of these reasons is the basis for the measurement, it must show a correlation with organoleptic deterioration if it is to be of value.

The organoleptic observations showed a much better correlation with time than with any chemical analyses. Of the chemical analyses, the peroxide content gave the best correlation with flavor score but even this showed no correlation with flavor score on the March fat at 4°. The tocopherol content showed a highly significant correlation with flavor on the June fat at 40° but otherwise no correlation. The correlation of the carbonyl content showed a probability of 0.10 or less at all temperatures on the June fat so that considering only this fat one might think it was second (to peroxide content) as a measure of deterioration. However, on the March fat the probability exceeded 0.10 at all temperatures except 25°. The iodine and hydrogenation values on the basis of this study do not appear to have value as means of detecting impending deterioration.

IV. SUMMARY AND CONCLUSIONS

A. Hydrogenation Method

1. Need for new method.

Hydrogenation was chosen as a method of analysis to be developed for a study of oxidizing fat because the rate of oxidation is considered to be related to the degree of unsaturation, a quality which is not considered to be accurately measured by the existing iodine number methods and because the hydrogenation value might be expected to increase upon oxidation while the iodine value would be expected to either remain constant or to decrease (experience generally has shown it to decrease). The divergence of these values as well as their initial values and ratios should give information regarding the course of oxidation. A review of the literature failed to reveal any satisfactory method which would be sensitive enough for the study desired.

2. Equipment

The Warburg apparatus commonly used in respiration studies was chosen as the basic equipment for the determination of hydrogenation values because it provides accurate

temperature control, is easily adapted to multiple simultaneous determinations and could be expected to provide a method with a high degree of reading accuracy. Several modifications were found to be necessary, chief of which was the elimination of all flexible tubing and providing of an all-glass system. Although mercury was used as a manometer fluid, it was necessary to have a layer of propionic acid above the mercury to prevent the fouling of the manometer capillary by lubricants being carried from the ground joints by the surging action of the mercury.

An all-glass system was designed and constructed for the purposes of generating and purifying hydrogen and of evacuating and filling the reaction flask-manometer combinations. Hydrogen, generated from zinc and sulfuric acid, was purified by silver nitrate and alkaline sodium stannite, dried with indicating drierite and phosphoric anhydride and finally passed through a trap immersed in liquid nitrogen.

3. Catalyst

A platinum oxide catalyst (Adams) was used. Because of the difficulties encountered in the dispersion of this catalyst when used in the pure form, it was prepared on a carrier. In the method finally adopted, the preparation contained not more than 3 per cent of active catalyst on 200 mesh Celite. To obtain a more uniform catalyst preparation, the fusion of

the platinum salt with sodium nitrate was carried out in a muffle furnace with controlled temperature. It was felt that a more active catalyst was obtained if the reduction of the platinum oxide in the reaction flask occurred in contact with a small amount of fat.

4. Results on methyl esters of unsaturated fatty acids

Hydrogenation of methyl oleate, methyl linoleate and methyl linolenate gave values very closely approximating the theoretical values. The results (as submitted by the supplier of the esters) of spectrophotometric analyses of the samples showed some slight amounts of impurities. Although the supplier of the samples reported Wijs iodine values agreeing quite closely with theoretical values, the values obtained in this study by the Rosenmund-Kuhnenn iodine value method was approximately 8 to 10 per cent below the theoretical values on the polyunsaturated esters. The reason for this is not known. It is apparently not a function of reaction time.

5. Results on fats direct from -25° storage

Throughout the development and use of the hydrogenation method, there was consistently noticed a difference between the hydrogenation iodine value and the iodine value (Rosenmund-Kuhnenn method) on fats taken direct from storage at -25°. The hydrogenation value was always higher but the magnitude of

the difference varied from one fat to another. While this difference may have its origin in variations among the polyunsaturated glyceride esters and thus be similar to that noted with the methyl esters, it is believed that other factors may play a part here. Unless a greater portion of the polyunsaturated compounds fail to react here than with the methyl esters, there is apparently not enough linoleate and linolenate in milk fat to account for differences of the magnitude found.

There was good agreement among the values obtained on fats direct from -25° storage although even in this respect there was a difference between two fats. For a March fat with a hydrogenation iodine value averaging 33.25 for eight determinations, the values covered a range of 0.09 unit with a standard deviation of 0.028 unit. A June fat with an average value of 40.68 covered a range of 0.23 unit; the standard deviation was 0.083 unit.

B. Oxidation Studies

1. Methods used for study

In addition to organoleptic evaluation of flavor, chemical analyses were made to determine iodine and hydrogenation iodine values and peroxide, carbonyl and tocopherol contents. A late winter fat and an early summer fat were selected for study to enable a comparison of the progress of

oxidation in fats produced at different seasons of the year. Aliquots of these two fats were stored at four different temperatures. Analyses were performed at intervals dependent upon the temperature of storage and increasing in frequency as the change in analytical values became more rapid.

2. General trends

The hydrogenation iodine value and the peroxide and carbonyl contents generally tended to increase while the iodine value and the numerical flavor score decreased. At the lower temperatures, tocopherol appeared to increase but decreased at the higher temperatures. In most cases the correlations of these changes with time were not significant. Disregarding the lowest temperature (where the samples were analysed only four times) the only case of significance at all temperatures was that of flavor score versus time, and that only with the June fat.

3. Changes in tocopherol content

The reason for the apparent increase in tocopherol content at the lower temperatures has not yet been determined. It is not thought that tocopherol is actually formed in the fat in vitro but apparently some substance not originally present in a form capable of reducing ferric iron to ferrous iron is produced during storage or is converted to a form

capable of performing this reduction. This may be an aldehyde which is initially formed at a greater rate than the tocopherol is oxidized at the lower temperature. As the temperature is increased, the oxidation of the tocopherols may be accelerated more than the formation of the aldehyde. However, it is believed that the rapid production of aldehydes at the higher temperatures would cause an apparent increase in the tocopherols (rather than the decrease observed) if this were the course of the reactions.

4. Carbonyl content and flavor development

As determined by organoleptic observations, the June fat deteriorated more rapidly than did the March fat. The rate of production of carbonyl compounds in the March fat exceeded that in the June fat; this would seem to constitute some evidence that the compounds responsible for the oxidized flavor are not carbonyl compounds as has often been thought. Alternatively it may indicate that, even though the flavor compound is a carbonyl compound, not all carbonyl compounds are produced at the same rate. This latter idea seems more logical.

5. Relationship of hydrogenation iodine value to iodine value

Throughout this portion of the study differences between the hydrogenation value and the iodine value persisted but were not of the same magnitude in the two fats, being about 0.39 and 1.37 (g. iodine per 100 g. fat) for the March and June fats respectively. The difference showed a slight tendency to increase during sample storage but temperature seemed to have no effect on the rate of change. Neither the increase in hydrogenation value, the decrease in iodine value nor the increasing difference showed a consistently significant correlation with any other variable. This study failed to reveal the cause of the difference and further study concerning this should be made.

6. Effect of temperature

As would be expected, increases in temperature accelerated the rates of change in the analytical values. However, the thermal coefficients were not uniform for the various reactions nor between fats for the same reaction. This indicates that the courses of the oxidation reactions are not the same at the higher temperatures as they are at the lower ones. Therefore, while accelerated reactions studied at higher temperatures may give information of value, it cannot show exactly what will happen at the lower temperatures

normally used for the storage of dairy products (and other fat containing materials).

7. Differences between fats

Experiences of the dairy industry in general have been that a winter fat develops an oxidized flavor more rapidly than a summer fat. This has been attributed to a lessened content of tocopherol, the only fat soluble antioxidant considered to be present in the milk fat. The initial contents of tocopherol in the two fats studied would indicate that the June fat should be better protected against oxidation, the tocopherol content of the June fat being slightly more than twice that of the March fat. On the other hand, the greater degree of unsaturation of the June fat might be expected to render it more susceptible to oxidation. In contrast to the results generally experienced in industry for dairy products (cf. milk) the June fat did deteriorate more rapidly than the March fat. Concurrently there was a greater rate of destruction of the tocopherol in the March fat.

8. Prediction of shelf life of fats

The lack of significant correlations between the results of the chemical analyses and the organoleptic observations would seem to indicate that none of the chemical methods employed in this study is reliable either as a measure of the

degree of deterioration having already occurred or as a tool (used alone) in predicting the useful shelf life of a fat.

9. Hypotheses presented

From a study of the data obtained, two alternative hypotheses concerning the relationships among peroxide content, tocopherol content and flavor were presented. In both hypotheses, it was considered that free radicals produced in the oxidation process may take part in three types of reactions, namely (a) reactions producing peroxides (and through decomposition of these, the carbonyl compounds), (b) chain termination reactions related to tocopherol oxidation, and (c) reactions producing compounds responsible for oxidized flavor. In one hypothesis, the flavor reaction is considered chain propagating while in the other it is chain terminating. It is felt that the relative rates of these three types of reactions may differ from one fat to another and may be responsible for such observed occurrences as a greater tocopherol degradation for the formation of a given amount of peroxide in the June than in the March fat and a greater rate of tocopherol degradation in the March fat than in the June fat.

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VI. ACKNOWLEDGEMENTS

The writer wishes to express his appreciation and thanks to Dr. E. W. Bird who suggested the problem and under whose guidance the work was carried out. Thanks are also due Dr. Bird for construction of a portion of the equipment used and for his fine counselling in the techniques of glass-blowing. The writer is indebted to Dr. A. F. Tamsma and Dr. V. H. Nielsen for the carbonyl determinations and to Miss Dorothy Littig for the tocopherol determinations reported in this work. The assistance and encouragement given by Professor C. A. Iverson and the Dairy Industry Department of Iowa State College are gratefully acknowledged. The assistance of the Quartermaster Food and Container Institute for the Armed Forces, which supported a portion of this work, is likewise acknowledged. Appreciation is also expressed to Dr. P. L. Kelly of the Department of Dairy Husbandry, University of Nebraska for allowing the writer time for completion of this thesis.

VII. APPENDIX

Table 23

Analytical Results on March Fat Stored at -25°

| Days in storage | Hydrogenation iodine value | Iodine value | Peroxide contents ^a | Carbonyl content ^b | Tocopherol contents | Organoleptic flavor score |
|-----------------|----------------------------|--------------|--------------------------------|-------------------------------|---------------------|---------------------------|
| 0 | 33.25 | 32.91 | 0.109 | 0.022 | 14.24 | 10.00 |
| 18 | 33.10 | 32.73 | 0.093 | 0 | 18.66 | 9.50 |
| 36 | 33.31 | 32.85 | 0.116 | 0.057 | 16.45 | 9.50 |
| 54 | 33.33 | 32.65 | 0.126 | 0 | 16.77 | 8.50 |

^ameq. Peroxide per kg. fat.
^bmmol. Carbonyl per kg. fat.
^cmcg. Tocopherol per g. fat.

Table 2/4
Analytical Results on March Fat Stored at 4°

| Days in storage | Hydrogenation iodine value | Iodine value | Peroxide content ^a | Carbonyl content ^b | Tocopherol content ^c | Organoleptic flavor score |
|-----------------|----------------------------|--------------|-------------------------------|-------------------------------|---------------------------------|---------------------------|
| 0 | 33.25 | 32.91 | 0.109 | 0.022 | 14.24 | 10.00 |
| 12 | 33.29 | 32.90 | 0.147 | 0 | 14.62 | 6.75 |
| 24 | 33.17 | 32.90 | 0.234 | 0.115 | 14.25 | 8.00 |
| 36 | | 32.83 | 0.561 | 0.057 | 15.50 | 9.50 |
| 42 | 33.28 | 32.85 | 0.392 | 0.074 | 16.77 | 8.00 |
| 48 | 33.27 | 32.77 | 0.091 | 0.156 | 16.93 | 8.75 |
| 54 | 33.30 | 32.64 | 0.374 | 0.052 | 15.58 | 6.75 |
| 59 | 33.34 | 32.88 | 0.393 | 0.118 | | 5.75 |

^ameq. Peroxide per kg. fat.
^bmmol. Carbonyl per kg. fat.
^cmcg. Tocopherol per g. fat.

Table 25

Analytical Results on March Fat Stored at 25°

| Days in storage | Hydrogenation iodine value | Iodine value | Peroxide contents | Carbonyl content ^b | Tocopherol contents | Organoleptic flavor score |
|-----------------|----------------------------|--------------|-------------------|-------------------------------|---------------------|---------------------------|
| 0 | 33.25 | 32.91 | 0.109 | 0.022 | 14.24 | 10.00 |
| 6 | 33.24 | 32.87 | 0.162 | 0.031 | 13.65 | 8.75 |
| 12 | 33.27 | 32.83 | 0.406 | 0.159 | 13.19 | 7.50 |
| 15 | 33.25 | 32.71 | 0.406 | 0.119 | 15.48 | 8.00 |
| 18 | 33.11 | 32.87 | 0.554 | 0.026 | 18.84 | 7.25 |
| 21 | 33.20 | 32.91 | 0.907 | 0.046 | 13.79 | 7.25 |
| 24 | 33.17 | 32.92 | 0.622 | 0.050 | 17.50 | 7.50 |
| 27 | 33.24 | 32.89 | 1.086 | 0.098 | 11.04 | 7.25 |
| 30 | 33.28 | 32.89 | 1.041 | 0.137 | 14.45 | 8.25 |
| 33 | 33.31 | 32.82 | 1.515 | 0.170 | 16.33 | 6.75 |
| 36 | 33.24 | 32.89 | 0.678 | 0.315 | 18.73 | 6.50 |
| 39 | 33.26 | 32.94 | 0.580 | 0.165 | 16.29 | 6.50 |
| 42 | 33.27 | 32.87 | 0.783 | 0.157 | 14.72 | 8.00 |
| 45 | 33.35 | 32.79 | 1.422 | 0.279 | 12.58 | 6.75 |
| 48 | 33.17 | 32.78 | 0.469 | 0.154 | 14.72 | 7.50 |
| 51 | 33.27 | 32.72 | 1.684 | 0.150 | 13.84 | 6.50 |
| 54 | 33.24 | 32.70 | 1.529 | 0.148 | 17.09 | 6.00 |
| 57 | 33.34 | 32.78 | 1.501 | 0.092 | 14.14 | 5.00 |
| 59 | 33.25 | 32.83 | 2.287 | 0.157 | 13.18 | 5.25 |

ameq. Peroxide per kg. fat.
 mmol. Carbonyl per kg. fat.
 cmol. Tocopherol per g. fat.

Table 26

Analytical Results on Marsh Fat Stored at 40°

| Days in storage | Hydrogenation iodine value | Iodine value | Peroxide content ^a | Carbonyl content ^b | Tocopherol contents | Organoleptic flavor score |
|-----------------|----------------------------|--------------|-------------------------------|-------------------------------|---------------------|---------------------------|
| 0 | 33.10 | 32.73 | 0.093 | 0 | 18.66 | 9.50 |
| 2 | 33.15 | 32.81 | 0.362 | 0.092 | 16.45 | 8.75 |
| 4 | 33.18 | 32.93 | 0.737 | 0.013 | 15.06 | 7.50 |
| 5 | 33.28 | 32.85 | 0.838 | 0.059 | 13.87 | 7.50 |
| 6 | 33.17 | 32.89 | 0.904 | 0.177 | 15.03 | 7.75 |
| 7 | 33.31 | 32.97 | 1.431 | 0.243 | 12.93 | 6.50 |
| 8 | 33.22 | 32.94 | 1.145 | 0.067 | 18.81 | 6.50 |
| 9 | 33.19 | 32.93 | 1.538 | 0.154 | 11.03 | 7.25 |
| 10 | 33.33 | 32.92 | 1.651 | 0.124 | 11.99 | 7.00 |
| 11 | 33.19 | 32.98 | 1.984 | 0.256 | 10.96 | 5.75 |
| 12 | 33.35 | 32.90 | 2.147 | 0.234 | 12.90 | 8.00 |
| 13 | 33.21 | 32.89 | 1.982 | 0.172 | 11.11 | 7.50 |
| 14 | 33.28 | 32.86 | 1.655 | 0.195 | 12.88 | 6.25 |
| 15 | 33.31 | 32.99 | 3.066 | | | 6.50 |
| 16 | 33.19 | 32.89 | 2.821 | | | 5.00 |
| 17 | 33.20 | 32.90 | 1.800 | 0.211 | 16.29 | 5.50 |
| 18 | 33.20 | 32.85 | 2.713 | 0.333 | 12.43 | 3.50 |
| 19 | 33.29 | 32.87 | 3.683 | 0.487 | 8.81 | 3.00 |

^ameq. Peroxide per kg. fat.
^bmmol. Carbonyl per kg. fat.
 cmg. Tocopherol per g. fat.

Table 27

Analytical Results on June Fat Stored at -25°

| Days in storage | Hydrogenation iodine value | Iodine value | Peroxide content ^a | Carbonyl content ^b | Tocopherol content ^c | Organoleptic flavor score |
|-----------------|----------------------------|--------------|-------------------------------|-------------------------------|---------------------------------|---------------------------|
| 0 | 40.68 | 39.38 | 0.174 | 0.013 | 30.01 | 9.33 |
| 18 | 40.50 | 39.36 | 0.147 | 0.053 | 35.25 | 9.50 |
| 36 | 40.68 | 39.31 | 0.128 | 0.056 | 33.05 | 9.50 |
| 54 | 40.72 | 39.16 | 0.154 | 0 | 31.85 | 9.00 |

^ameq. Peroxide per kg. fat.
^bmmol. Carbonyl per kg. fat.
^cmg. Tocopherol per g. fat.

Table 28

Analytical Results on June Fat Stored at 40°

| Days in storage | Hydrogenation iodine value | Iodine value | Peroxide content ^a | Carbonyl content ^b | Tocopherol contents | Organoleptic flavor score |
|-----------------|----------------------------|--------------|-------------------------------|-------------------------------|---------------------|---------------------------|
| 0 | 40.68 | 39.38 | 0.174 | 0.013 | 30.01 | 9.33 |
| 12 | 40.72 | 39.30 | 0.150 | 0 | 30.82 | 8.75 |
| 24 | 40.70 | 39.44 | 0.216 | 0.072 | 31.23 | 9.50 |
| 36 | 40.70 | 39.38 | 1.181 | 0.085 | 32.68 | 4.50 |
| 42 | 40.74 | 39.35 | 0.508 | 0.135 | 32.26 | 4.50 |
| 48 | 40.64 | 39.35 | 0.475 | 0.166 | 32.68 | 5.50 |
| 54 | 40.72 | 39.23 | 0.696 | 0.091 | 33.63 | 3.75 |
| 59 | 40.73 | 39.28 | 0.853 | 0.175 | 30.94 | 2.50 |

^ameq. Peroxide per kg. fat.
^bmmol. Carbonyl per kg. fat.
 cmcg. Tocopherol per g. fat.

Table 29

Analytical Results on June Fat Stored at 25°

| Days in storage | Hydrogenation iodine value | Iodine value | Peroxide content ^a | Carbonyl content ^b | Tocopherol content ^c | Organoleptic flavor score |
|-----------------|----------------------------|--------------|-------------------------------|-------------------------------|---------------------------------|---------------------------|
| 0 | 40.68 | 39.38 | 0.174 | 0.013 | 30.01 | 9.33 |
| 6 | 40.82 | 39.31 | 0.244 | 0.019 | 29.18 | 8.25 |
| 12 | 40.75 | 39.32 | 0.277 | 0.027 | 30.17 | 8.50 |
| 18 | 40.53 | 39.30 | 0.780 | 0.191 | 32.89 | 7.75 |
| 21 | 40.59 | 39.42 | 0.691 | 0.091 | 32.84 | 6.75 |
| 24 | 40.58 | 39.43 | 0.635 | 0.011 | 34.81 | 8.75 |
| 27 | 40.66 | 39.37 | 1.086 | 0.109 | 23.03 | 6.50 |
| 30 | 40.65 | 39.33 | 1.219 | 0.103 | 28.24 | 7.50 |
| 33 | 40.69 | 39.38 | 2.773 | 0.170 | 27.63 | 3.50 |
| 36 | 40.55 | 39.37 | 1.342 | 0.283 | 39.52 | 4.25 |
| 39 | 40.73 | 39.42 | 1.802 | 0.208 | 28.78 | 3.00 |
| 42 | 40.76 | 39.34 | 1.047 | 0.180 | 28.63 | 2.00 |
| 45 | 40.80 | 39.24 | 1.794 | 0.348 | 28.83 | 2.00 |
| 48 | 40.65 | 39.31 | 2.251 | 0.199 | 26.89 | 3.00 |
| 51 | 40.77 | 39.14 | 2.013 | 0.165 | 28.33 | 1.50 |
| 54 | 40.78 | 39.16 | 1.039 | 0.202 | 33.52 | 5.50 |
| 57 | 40.70 | 39.35 | 1.086 | 0.058 | 29.62 | 1.50 |
| 59 | 40.61 | 39.33 | 2.102 | 0.155 | 31.44 | 1.75 |

^ameq. Peroxide per kg. fat.^bmmol. Carbonyl per kg. fat.^cmcg. Tocopherol per g. fat.

Table 30

Analytical Results on June Fat Stored at 40°

| Days in storage | Hydrogenation iodine value | Iodine value | Peroxide content ^a | Carbonyl content ^b | Tocopherol content ^c | Organoleptic flavor score |
|-----------------|----------------------------|--------------|-------------------------------|-------------------------------|---------------------------------|---------------------------|
| 0 | 40.68 | 39.31 | 0.128 | 0.056 | 33.05 | 9.50 |
| 2 | 40.69 | 39.39 | 0.164 | 0.163 | | 9.50 |
| 4 | 40.60 | 39.37 | 0.309 | 0.234 | 32.01 | 9.50 |
| 6 | 40.75 | 39.39 | 0.460 | 0.101 | 32.38 | 7.75 |
| 8 | 40.77 | 39.34 | 0.726 | 0.147 | 32.05 | 6.50 |
| 10 | 40.65 | 39.46 | 1.954 | 0.245 | 31.52 | 6.00 |
| 11 | 40.79 | 39.22 | 1.693 | 0.339 | 30.17 | 5.00 |
| 12 | 40.70 | 39.38 | 0.630 | 0.141 | 32.89 | 5.50 |
| 13 | 40.68 | 39.32 | 0.481 | 0.280 | 32.47 | 3.50 |
| 14 | 40.60 | 39.37 | 1.458 | 0.199 | 29.97 | 5.00 |
| 16 | 40.77 | 39.23 | 0.819 | 0.135 | 32.58 | 5.50 |
| 18 | 40.81 | 39.15 | 1.334 | 0.039 | 30.67 | 4.00 |
| 20 | 40.67 | 39.18 | 2.757 | 0.304 | 29.62 | 3.00 |
| 22 | 40.73 | 31.31 | 2.384 | 0.170 | 29.62 | 2.50 |
| 24 | 40.77 | 39.24 | 3.435 | 0.394 | 26.09 | 1.25 |
| 25 | 40.70 | 39.28 | 2.105 | 0.169 | 29.72 | 1.50 |

^ameq. Peroxide per kg. fat.^bmmol. Carbonyl per kg. fat.^cmg. Tocopherol per g. fat.

Table 31

Coefficients of Regression Together with
Degree of Significance of Correlation Coefficients

| Variables, dependent - independent | Temp. C. | Regression coefficients and degree of significance ^a of correlation coefficients on | | Denominational units of regression |
|--|-------------|--|-----------|--|
| | | March fat | June fat | |
| Hydrogenation iodine value - time | -25 | / .0025 o | / .0017 o | Hydrogenation iodine value units per day |
| | 4 | / .0012 o | / .0003 o | |
| | 25 | / .0010 o | / .0006 o | |
| | 40 | / .0049 o | / .0023 o | |
| Iodine value - time | -25 | - .0037 o | - .0039 p | Iodine value units per day |
| | 4 | - .0027 o | - .0016 o | |
| | 25 | - .0018 p | - .0019 p | |
| | 40 | / .0032 o | - .0062 s | |
| Peroxide content - time | -25 | / .0004 o | - .0004 o | μ eq. Peroxide per g. fat per day ^b |
| | 4 | / .0042 o | / .0117 p | |
| | 25 | / .0261 v | / .0293 v | |
| | 40 | / .1565 v | / .1073 v | |
| Carbonyl content - time | -25 | - .0001 o | - .0002 o | μ mol. Carbonyl ^c per g. fat per day |
| | 4 | / .0016 p | / .0028 v | |
| | 25 | / .0023 s | / .0031 s | |
| | 40 | / .0180 v | / .0043 o | |
| Tocopherol content - time | -25 | / .0299 o | / .0184 o | mcg. Tocopherol per g. fat per day |
| | 4 | / .0448 s | / .0400 p | |
| | 25 | - .0021 o | - .0114 o | |
| | 40 | - .2987 s | - .1856 v | |
| Flavor score - | -25 | - .0250 p | - .0055 o | Flavor score units per day |
| | 4 | - .0357 o | - .1200 v | |
| | 25 | - .0544 v | - .1417 v | |
| | 40 | - .2467 o | - .3388 v | |

^aDegree of significance is indicated as: o - no significance
p - possibly significant
s - significant
v - very significant

^b μ eq. Peroxide per g. fat is numerically equivalent to meq. peroxide per kg. fat.

^c μ mol. Carbonyl per g. fat is numerically equivalent to mmol. carbonyl per kg. fat.

Table 31 (continued)

| Variables, dependent - independent | Temp. °C. | Regression coefficients and degree of significance of correlation coefficients on | | Denominational units of regression |
|---|----------------------|---|---|---|
| | | March fat | June fat | |
| Hydrogenation iodine value - flavor score | -25 4 25 40 | -10.7299 o - 5.7977 o / 2.0423 o -17.0608 o | -2.0850 o -6.2137 o / .3062 o / 8.9022 o | Hydrogenation iodine value units per flavor score unit |
| Iodine value - flavor score | -25 4 25 40 | - .0310 o / .0188 o - .0125 p - .0124 s | - .0356 o - .0005 o - .0100 o - .0061 o | Iodine value units per flavor score unit |
| Peroxide content - flavor score | -25 4 25 40 | - .0031 o / .0857 o - .0610 o - .2396 v | - .0057 o / .1376 o - .0979 o - .4894 v | μ eq. Peroxide per g. fat per flavor score unit |
| Carbonyl content - flavor score | -25 4 25 40 | - .0056 o / .0254 o / .0019 o - .0308 v | / .0096 o / .0198 o - .0045 o - .0333 s | μ mol. Carbonyl per g. fat per flavor score unit |
| Tocopherol content - flavor score | -25 4 25 40 | / 7.2664 s / .1565 o / .0413 p / .0347 p | - .7433 o / .0160 o / .0012 o / .0108 o | mg. Tocopherol per g. fat per flavor score unit |
| Hydrogenation iodine value - iodine value | -25 4 25 40 | / .4170 o - .0027 o / .0453 o / .1271 v | -1.0813 o / .0930 o / .0796 v / .0583 s | Hydrogenation iodine value units per iodine value unit |
| Peroxide content - flavor score | -25 4 25 40 | / .0776 o - .1133 o / .3461 s / .2238 o | -2.2023 o - .0175 o / .0245 o - .0810 o | μ eq. Peroxide per g. fat per iodine value unit |
| Carbonyl content iodine value | -25 4 25 40 | / .0062 o / .0039 o / .0029 o - .0004 o | / .0056 o / .0019 o / .0025 o / .0085 o | μ mol. Carbonyl per g. fat per iodine value unit |

Table 31 (continued)

| Variables, dependent - independent | Temp. | Regression coefficients and degree of significance of correlation coefficients on | | Denominational units of regression |
|---|----------------------|---|---|--|
| | | March fat | June fat | |
| Tocopherol content - iodine value | -25 4 25 40 | - .0684 o - .0211 o - .0118 o - .0103 o | - .2607 o - .0036 o - .0073 o - .0070 o | mcg. Tocopherol per g. fat per iodine value unit |
| Hydrogenation iodine value - tocopherol content | -25 4 25 40 | / .1663 o / .0104 o / .0223 o - .0098 o | / .3484 o / .0142 o / .0082 o / .0165 s | Hydrogenation iodine value units per mcg. tocoph- erol per g. fat |
| Peroxide content - tocopherol content | -25 4 25 40 | - .0135 o - .0176 o - .3830 v - .4684 o | - .0335 o - .1086 s - .1989 v - .3019 v | μ eq. Peroxide per mcg. tocopherol |
| Carbonyl content - tocopherol content | -25 4 25 40 | / .0176 o - .0031 o - .0281 p - .0599 o | - .1093 p - .0182 s - .0207 p - .0156 p | μ mol. Carbonyl per mcg. tocoph- erol |
| Hydrogenation iodine value - peroxide content | -25 4 25 40 | - 1.1663 o - .0310 o - .1561 o / .8746 o | / 4.6504 o - .2682 o / .2747 o / .5312 v | Hydrogenation iodine value units per μ eq. peroxide per g. fat |
| Carbonyl content - peroxide content | -25 4 25 40 | / .0328 o - .1580 o - .2091 o / .5151 s | - .5409 o - .1878 o - .5566 s - .4183 s | μ mol. Carbonyl per μ eq. peroxide |
| Hydrogenation iodine value - carbonyl content | -25 4 25 40 | - .0234 o - .1291 o - 3.2201 p / 5.3987 o | - .0321 o - 1.3945 o - 1.6622 o - 4.9253 o | Hydrogenation iodine value units per μ mol. carbonyl per g. fat |
| (Hydrogenation iodine value minus iodine value) - time | -25 4 25 40 | / .1571 o - .0857 o - .2571 o / .4425 o | / .1508 o - .1779 o - .4188 o - .1340 o | g. Iodine per 100 g. fat per day |